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THE AGGLUTINATION AND SEQUESTRATION OF IMMATURE RED CELLS

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THE reticulocyte has been widely employed as a model cell for the study in vitro of certain metabolic processes. This is because it is a discrete cell which can be safely manipulated, which possesses the enzymatic apparatus for aerobic as well as anaerobic glycolysis,[1] and which carries out the active synthesis of proteins[2] and probably of fats.[3] The metabolic activity of reticulocytes, as judged by the rate of iron incorporation into hemoglobin, varies directly with their morphologic immaturity and differs from species to species. The circulating immature reticulocyte in man takes up iron at less than one tenth the rate estimated for the average nucleated red cell of the human bone marrow.[4] In contrast, the uptake of iron by the immature reticulocyte of rabbit blood is approximately one half that of the average nucleated red cell of the rabbit bone marrow.[5] During studies of iron metabolism in reticulocytes from various species it was noted that these immature red cells possessed properties usually encountered in mature red cells coated with antibodies. Further studies were conducted in which the reticulocyte was employed for exploring certain of these special properties associated with cellular immaturity (in the red cell series, at least). A preliminary report of some of the experiments described below was published.[6]

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METHODS

Hematologic Studies.—Peripheral blood hemoglobin, red cell concentrations, and hematocrit levels were determined by standard techniques.[7]

Permanent preparations of reticulocytes were made with alcoholic solutions of brilliant cresyl blue, counterstained with Wright's stain. Supravital staining with brilliant cresyl blue was employed for the identification of reticulocytes in red cell suspensions after their experimental manipulation. Blood smears from various organs for the enumeration of reticulocytes were obtained by removing a tangential slice from the organ and briefly pressing a cover slip stained with brilliant cresyl blue on the cut surface. With either peripheral or visceral blood, reticulocyte counts were based upon inspection of at least 1,000 red cells.

The hemoglobin content of rat spleen tissue was determined by blending each spleen in 50 ml. of water which contained 0.001M $K_3Fe(CN)_6$ and 0.001M KCN. The blended suspension was filtered through No. 42 Whatman filter paper and measurements were made by the quantitative benzidine technique.[7] As with the pyridine hemochromogen method,[8] the comparatively small amounts of tissue heme present in the spleen are included by this measurement. The hemoglobin content of spleens of 16 normal rats, with an average body weight of 274 grams and an average spleen weight of 0.91 grams, was 18.9 ± 7.1 mg. (mean ± 1 S. D.). The mean value is remarkably close to that obtained by Giblett and associates[9] who used the pyridine hemochromogen method.

Red cell agglutination was determined in 3 ways. *Frank agglutination* (autoagglutination) was defined as the existence of red cell clumping when blood was diluted in large (approximately 100) volumes of saline. *Increased agglutinability* was assessed by exposing blood to 5 per cent solutions of polyvinylpyrrolidone (PVP)* in isotonic phosphate buffer, pH 7.4, and subsequently diluting the suspension in saline, as described elsewhere.[10] In comparative studies of the agglutinability in PVP of human, rabbit, and rat red cells, consistent species differences were noted. As described previously,[10] human red cells form rouleaux in PVP which has a relatively low molecular weight, as indicated by its viscosity grade of K-30. Not until the molecular weight and viscosity of PVP are much higher (K-50 to K-62) is a small amount of frank agglutination, which persists after dilution with saline, produced in normal human red cells. Rabbit red cells formed rouleaux more readily with the K-30 grade of PVP, and actual agglutination was produced by the K-44 grade, a phenomenon never observed with normal human red cells. In contrast, rat red cells formed very poor rouleaux at any grade of PVP and agglutination was not observed even in the presence of the K-50 grade. Consequently, in investigating red cells for increased agglutinability, the K-44 preparation of PVP was used with human cells, K-30 with rabbit cells, and K-50 with rat cells. The presence of *protein on the red cell surface* was determined by the agglutinability of red cells in Coombs sera.[11] Coombs sera applicable to various species were produced as described by Emerson and associates.[12] Coombs serum for use with human red cells was obtained from immunized rabbits, sera for use with rat red cells were obtained from both rabbits and guinea pigs, and sera for use with rabbit red cells were obtained from both rats and guinea pigs. In certain instances Coombs serum for use with human red cells was absorbed free of at least part of its antiprotein activity by being incubated before use with various volumes of human serum or of solutions in saline of certain relatively pure fractions (>97 per cent purity by electrophoretic analysis) of human serum protein, i. e., albumin (Cohn Fraction V), gamma globulin (Cohn Fraction II-1, 2), and transferrin crystallized from Cohn Fraction IV-7.[13] The amount of each protein fraction employed in the absorption of Coombs sera was determined in advance to be the amount which causes maximum precipitation of antigen-antibody complexes. The amounts actually employed to neutralize the respective antibodies in 1 ml. of Coombs serum varied from 1 to 10 mg. of protein.

Test tubes which contained 5 per cent suspensions of thrice washed red cells and equal volumes of Coombs serum were incubated at 37° C. with gentle agitation for periods up to

*Schenley Laboratories, Inc., New York, N. Y.

2 hours. Intensity of agglutination was estimated semiquantitatively (0 to 4+ agglutination) on the basis of microscopic examination.

Animals.—In those experiments which involved the use of rats, parasite-free males, weighing 290 ± 50 grams, of either the Lobund or Charles River CD (cesarean-delivered) albino strains were used. The male New Zealand rabbits employed weighed 2.4 ± 0.4 kilograms. Splenomegaly in rats was produced with methylcellulose* after the method of Palmer and associates.[14] Three milliliters of a 2.5 per cent solution was injected intraperitoneally twice a week. Turpentine abscesses in rats were produced by injections of 0.5 ml. of turpentine into the retrofemoral muscles.

Radioactive Iron Studies.—Radioactive iron was employed in the form of $Fe^{59}Cl_3$.† Fe^{59} was bound to rat plasma transferrin by the slow addition of 1 mcg. of Fe^{59} to each milliliter of fresh heparinized rat plasma, obtained in acid-cleaned glassware, and the mixture was incubated for 1 hour at room temperature. Into each animal so studied, 0.5 mcg. (from 0.5 to 1.5 μc) of Fe^{59} and approximately 1 mcg. of the nonradioactive plasma iron was injected by tail vein. The injected rats were given 1 or 2 mg. of heparin intravenously immediately prior to death by decapitation. Blood and tissue samples weighing 2 grams or less were counted in a well-type scintillation counter in which the apparent activity of samples was not significantly affected by variations within total volumes not exceeding 2 ml.

RESULTS

Behavior of Reticulocytes in Vitro.—

1. *Agglutinability of reticulocytes in man, rabbit, and rat:* The reticulocytes of normal human, rabbit, and rat blood showed no tendency to clump spontaneously or during centrifugation.

In man. Reticulocyte agglutination before or after centrifugation was not evident in blood which contained increased reticulocyte concentrations from patients with hemolytic anemia or from patients with pernicious anemia who were responding to therapy. When such reticulocyte-rich blood was exposed to 5 per cent PVP (K-44) or to Coombs serum (Fig. 1), however, agglutinates formed which consisted almost entirely of reticulocytes. The size of these reticulocyte clumps increased in proportion to the concentration of reticulocytes and the period of incubation. Under low power magnification red cell suspensions which contained only slightly increased numbers of reticulocytes would be interpreted by the usual criteria as giving negative or only weakly positive readings in the PVP test or the Coombs test. A quantitative assessment of the intensity of agglutination was quite difficult because a minor population of (reticulated) cells was being dealt with. Furthermore, the clumps of reticulocytes were partially dispersed by shaking or by the mechanical pressure of cover slips. If judged by the size, rather than the frequency, of the clumps, however, agglutination was in some instances sufficiently strong to be read by the usual criteria as 2+ or 3+. As shown in Fig 1, *B*, when nucleated red cells were present they adhered to reticulocytes as well as to each other when exposed to Coombs serum. Unlike antibody-coated red cells, which usually agglutinate more strongly in PVP than in Coombs serum, reticulocytes were

*Methocel, viscosity 400 c.p.s., The Dow Chemical Co., Midland, Mich.
†Abbott Laboratories, North Chicago, Ill.

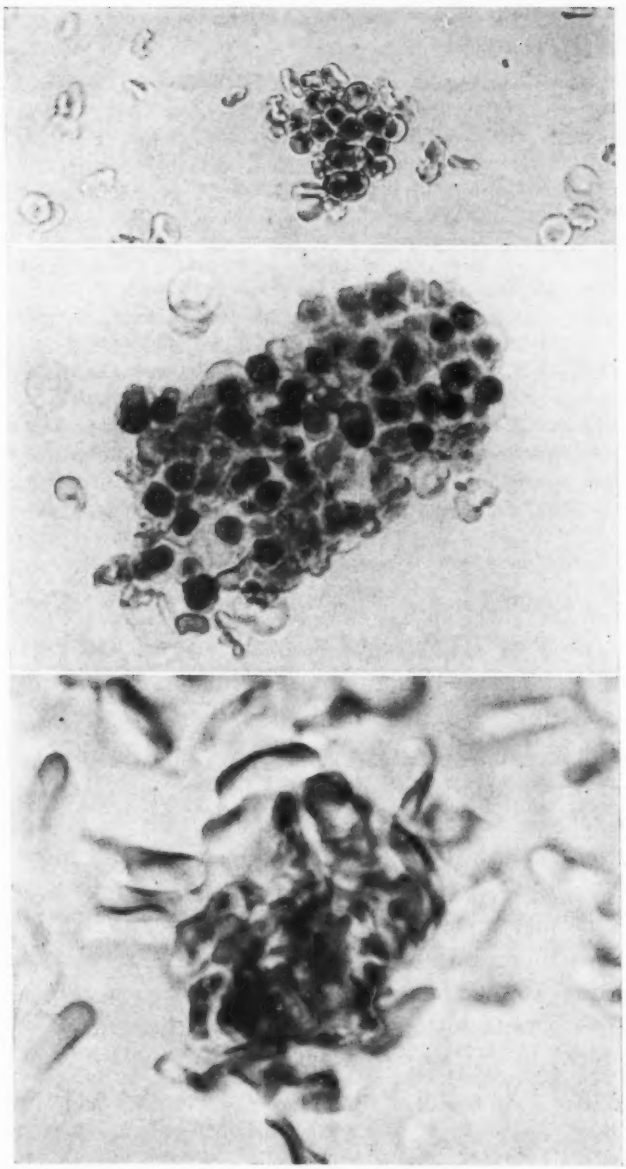

Fig. 1.—Reticulocyte agglutination produced by Coombs serum. *A*, On gross visual inspection the red cells of this patient with lymphatic leukemia and a hemolytic anemia did not appear to be agglutinated by Coombs serum. However, with microscopy sizable clumps were seen to be scattered throughout the suspension, and, on supravital staining with brilliant cresyl blue, virtually all of the clumped cells were found to be reticulocytes, as shown here, and almost all of the reticulocytes were in clumps. *B*, Blood was derived from a child with Cooley's anemia, with 4.8 per cent reticulocytes and 55,000 nucleated red cells per cubic millimeter. After incubation of the washed red cells with Coombs serum, almost all of the immature red cells were clumped, whereas almost all of the mature red cells were freely suspended. The large aggregate shown here consisted mostly of reticulocytes, the mottled and darkened cells without nuclei, intermixed with many normoblasts, and a few leukocytes. *C*, A higher power view of a typical reticulocyte clump produced by Coombs serum, in a suspension of red cells from another patient with Cooley's anemia. Although a uniform focus was not possible, the dark reticulum precipitated by brilliant cresyl blue is evident in the centrally agglutinated, but not in the unagglutinated cells.

clumped more strikingly in Coombs serum. Specific agglutinability of reticulocytes by Coombs serum was found in 6 of 10 patients studied who suffered from a variety of disorders which manifested reticulocytosis (Table I). In 2 patients with hereditary spherocytosis and reticulocyte levels between 4 and 5 per cent, no agglutination of reticulocytes by Coombs serum was demonstrable. In the blood from 2 other patients with "autoimmune" hemolytic anemias, all of whose red cells were agglutinated by Coombs serum, the agglutinates of red cells were composed of random mixtures of reticulated and nonreticulated red cells.

TABLE I. AGGLUTINATION BY COOMBS SERUM OF RETICULOCYTES FROM VARIOUS PATIENTS

DIAGNOSIS	HEMOGLOBIN CONCENTRATION (GM. %)	RETICULOCYTE CONCENTRATION (%)	AGGLUTINATION BY COOMBS SERUM	
			RETICULOCYTES	MATURE RED CELLS
Normal (8)	14.6 ± 1.2	0.7 ± 0.4	0	0
Cooley's anemia	7.5	5.6	2+	0
Cooley's anemia	4.2	4.8	2+	0
Sickle cell anemia	7.2	14.8	3+	0
Lymphatic leukemia	6.5	7.9	2+	0
Folic acid deficiency in response	8.1	10.8	1-2+	0
Folic acid deficiency in response	6.5	11.9	3+	0
Acquired hemolytic anemia	8.2	42.0	4+	4+
Acquired hemolytic anemia	7.2	14.5	4+	4+
Hereditary spherocytosis	10.8	4.6	0	0
Hereditary spherocytosis	12.1	4.2	0	0

TABLE II. EFFECT OF PRELIMINARY INCUBATION OF COOMBS SERUM ON ITS ABILITY TO AGGLUTINATE HUMAN RETICULOCYTES

SOURCE OF RETICULOCYTES—DIAGNOSIS	AGGLUTINATION OF RETICULOCYTES					
	IN SALINE	IN COOMBS SERUM PREINCUBATED WITH:				
		SALINE	SERUM	γ-GLOBULIN	ALBUMIN	TRANSFERRIN
Cooley's anemia	0	2+	0	2+	2+	1+
Cooley's anemia	0	2+	0	2+	2+	1+
Sickle cell anemia	0	3+	0	3+	3+	0
Lymphatic leukemia	0	2+	0	2+	2+	Wk 1+
Folic acid deficiency in response	0	1-2+	0	1-2+	1+	0
Folic acid deficiency in response	Wk 1+	3+	0	2+	3+	1+
Normal*	0	1+	0	1+	1+	0

*Reticulocyte concentration artificially increased to 4.2 per cent by differential centrifugation.

In rabbits. Reticulocytes from rabbits with reticulocytosis evoked by previous bleeding or by administration of phenylhydrazine were occasionally found to be clumped immediately ex vivo; larger clumps frequently were apparent after centrifugation. Occasionally in severely anemic rabbits with marked reticulocytosis, reticulocyte agglutination after centrifugation was macroscopically visible. However, exposure of such rabbit reticulocytes to PVP (Fig. 2) or to an appropriate Coombs serum invariably produced reticulocyte agglutinates. The relation between reticulocyte levels and red cell

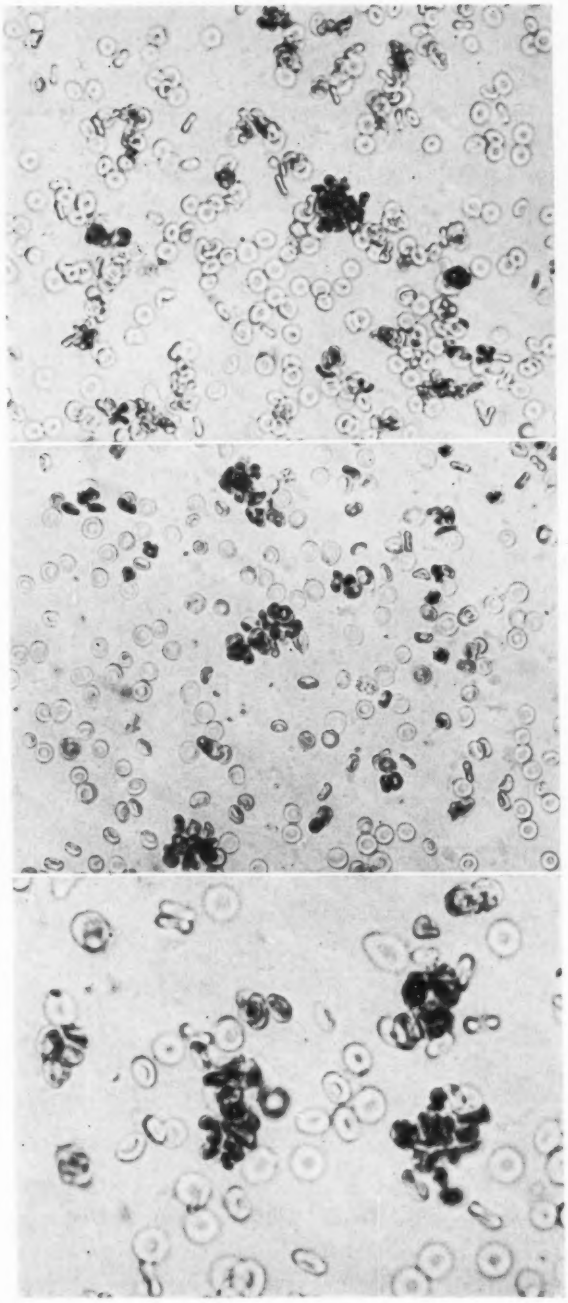

Fig. 2.—Reticulocyte agglutination produced by PVP. Rabbit blood drawn on the fourth day after 3 successive daily bleedings to 20 ml. per kilogram body weight showed grossly visible agglutination after exposure to PVP (K-30) or to rat antirabbit Coombs serum, whereas no such agglutination occurred in blood obtained prior to these bleedings. As shown here, supravital brilliant cresyl blue stained these very immature reticulocytes darkly, revealing that almost all clumped cells were reticulocytes and that the majority of reticulocytes were clumped.

agglutinability in rabbits is portrayed in Fig. 3. Similar changes appeared in 2 other rabbits that were injected with 25 mg. of acetylphenylhydrazine daily for 4 days. In both bled and phenylhydrazine treated rabbits, when reticulocyte concentrations reached 30 to 40 per cent, the red cells were as agglutinable as red cells previously exposed to an incomplete antibody.

In rats. Reticulocytes from bled rats were less agglutinable than those of rabbits. Like human reticulocytes, they occasionally formed small clumps during centrifugation, but strong clumping developed only after the addition of PVP or Coombs serum.

2. *Factors affecting the reaction between reticulocytes and Coombs serum:* In all 3 species, reticulocytes were agglutinated less strongly by undiluted preparations of the Coombs sera employed, than by from 1:10 to 1:50 dilutions of these sera, apparently a prozone effect. Reticulocyte agglutination was most pronounced when the cells were incubated with Coombs serum at 37° C.; there was less agglutination at 24° C. and no agglutination at 0.5° C. Sterile incubation of reticulocyte-rich blood at 37° C. for 24 hours diminished reticulocyte agglutinability in Coombs serum and in PVP besides diminishing the concentration of stainable reticulocytes by about 70 per cent. Incubation at 4° C. for a similar period did not diminish the agglutinability of reticulocytes or, as noted by Heath and Daland,[15] the concentration of reticulocytes. Incubation at 37° C. for 24 hours in the combined presence of 0.01M NaCN and 0.01M NaF did not reduce the number or agglutinability of reticulocytes.

The effect of prior absorption of Coombs serum with fractions of serum proteins was assessed in man, using reticulocyte-rich blood from 7 patients. Preliminary incubation of Coombs serum with whole human serum prevented the agglutination of reticulocytes by Coombs serum (Table II). Absorption of Coombs serum with either albumin or gamma globulin had no such blocking effect. Preincubation of Coombs serum with transferrin, however, consistently diminished, and in 3 instances, abolished its agglutinating effect. Efforts were made to obtain eluates from stroma derived from reticulocyte-rich red cells as described by Kidd.[16] Such eluates contained no substance which rendered normal mature red cells agglutinable by Coombs serum.

Behavior of Reticulocytes in Vivo.—

1. *The organ distribution of reticulocytes and of Fe^{59} in the rat:* The relative distribution of reticulocytes in blood derived from various organs was studied in 16 normal rats. The average reticulocyte concentration of blood obtained by tail puncture was 2.41 per cent. There was no significant difference in reticulocyte concentrations in blood obtained from the lungs, livers, or kidneys. Blood from the spleens of these rats revealed an average reticulocyte concentration of 3.78 per cent. The difference between reticulocyte levels in the spleen and peripheral blood is highly significant ($t = 3.68$, $p = <0.0025$).

The distribution of reticulocytes in various organs during a reticulocyte response to bleeding was followed in a group of 18 rats. Each was bled by heart puncture of 20 ml. blood per kilogram body weight and 1 ml. of rat plasma

labeled with 0.5 meg. of Fe^{59} was then injected through the same needle. Animals were then killed in threes at intervals of 4 hours, 1 day, 2 days, 3 days, 4 days, and 5 days, and certain organs were examined for reticulocyte concentrations and Fe^{59} content (Fig. 4). The reticulocyte levels of the peripheral (tail) blood and of blood obtained from the livers and from the lungs (not shown) increased slightly on both the first and second days after bleeding; a more marked rise occurred on the third day. Reticulocyte levels in the spleen,

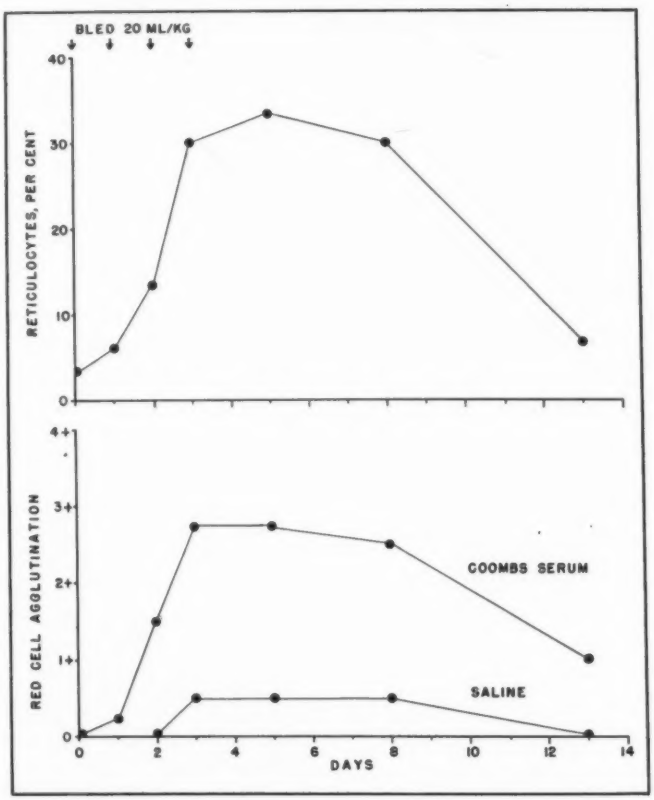

Fig. 3.—The agglutinability of rabbit red cells during the reticulocyte response to repeated bleeding. The reticulocyte levels and the degrees of agglutination indicated represent the average of 3 rabbits. Frank agglutination, evident in saline suspensions of cells (autoagglutination), was weakly present during the height of the reticulocyte responses. At this time the Coombs test was markedly positive because of the development of reticulocyte agglutinates. After the first few days the minor number of nonreticulated red cells involved in these clumps tended to increase, but the strength of agglutination diminished.

however, showed a moderate increase on the first day and further moderate increases daily thereafter, with reticulocyte concentrations averaging approximately twice those in the peripheral blood. Occasionally reticulocyte clumps were seen in blood smears taken from the spleen and liver. A few late normoblasts were seen in the spleen blood smears, but not until the third and fourth days after the bleeding. There were no early erythroid precursors in any of the smears examined.

Studies of the distribution of Fe^{59} in these bled rats (Fig. 4) showed a progressive decline in femoral marrow Fe^{59}, as the peripheral blood Fe^{59} rose in a reciprocal fashion (not shown). Hepatic Fe^{59} levels remained approximately constant, whereas splenic Fe^{59} activity reached a peak level 1 day after the bleeding, and then steadily fell, despite the rapidly increasing radioactivity of the perfusing blood. The rise in radioactivity in the spleen represented only about 2 per cent of the injected iron, however. Even if it is assumed that all of this radioactivity was derived from labelled reticulocytes, this indicates that no more than 2 per cent of newly released red cells were arrested in the spleen at any one time.

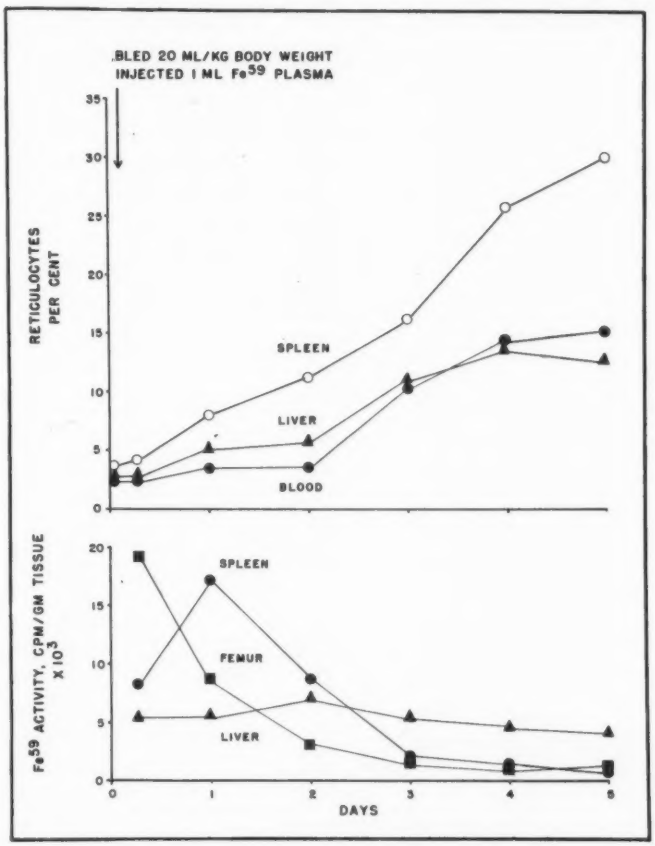

Fig. 4.—Distribution of reticulocytes and of radioactive iron in bled rats. The initial (zero time) reticulocyte levels represent averages from 16 unbled rats. Each of the other points represents average values for 3 bled rats: there were 18 bled rats, which were killed in groups of 3 at 6 different time intervals after bleeding. Reticulocyte levels (upper portion of figure) were distinctly higher in the spleen than elsewhere, despite the absence of splenic erythropoiesis. Note that the reticulocyte levels in the spleen showed striking increases 1 and 2 days after bleeding, before peripheral reticulocyte levels had risen appreciably. Spleen radioactivity (lower portion of figure) increased between 4 and 24 hours, and then rapidly declined after 24 hours to reach negligible levels by 3 days; this occurred despite the fact that peripheral blood radioactivity (not shown) continued to increase during the first 2 days and declined only slightly in the following 3 days. This transient rise in spleen Fe^{59} levels 1 day after bleeding and the early increase in spleen reticulocyte concentrations together suggest that a portion of the reticulocytes issued from the bone marrow were sequestered in the spleen.

2. *The distribution of Fe^{59}-labelled reticulocytes injected into rats:* To further investigate splenic trapping of reticulocytes, 2 rats which had undergone splenectomy were bled 30 ml. per kilogram body weight, and 1 day later 2 ml. of plasma which contained 2 mcg. of Fe^{59} was injected into each by tail vein. Twenty-four hours later, when blood Fe^{59} was present almost entirely in newly released reticulocytes, heparinized blood was drawn from these rats and pooled. In each of 4 normal rats 3 ml. of blood withdrawn by heart puncture was replaced through the same needle by 3 ml. of pooled blood which contained Fe^{59}-labelled reticulocytes from the animals which had undergone splenectomy. The recipient animals were killed 1 hour later. Analysis showed that an average of 94.0 per cent of the Fe^{59} was in the circulating blood and 2.3 per cent was in the spleen. Since the normal rat spleen contains about 0.7 per cent of the total body hemoglobin, this indicates that no more than 1.6 per cent of the injected Fe^{59}-labelled reticulocytes were trapped in the spleen at that time.

3. *Effect of splenectomy on reticulocyte levels in rats:* The effect of splenectomy on peripheral blood reticulocyte levels was assessed by bleeding 6 splenectomized and 6 sham-operated rats of 20 ml. blood per kilogram body weight by heart puncture; thereafter blood hemoglobin and reticulocyte levels were measured daily for 7 days. There were no significant differences between these 2 groups in either the levels of reticulocytes or the rises in hemoglobin concentration (Fig. 5).

4. *Effect of splenomegaly on reticulocyte levels in rats:* The effect of splenomegaly induced by methylcellulose on spleen reticulocyte levels was studied in 12 rats killed in pairs at 2 week intervals after the start of methylcellulose injections. The ratio of reticulocyte concentrations in spleen blood to peripheral blood averaged only 1.07. This is less than in normal rats or in bled rats with comparable peripheral blood reticulocyte levels. As in the other rats studied, the reticulocyte levels of livers and lungs were similar to those of the peripheral blood.

The effect of inflammation upon reticulocyte distribution was studied in 4 rats injected intramuscularly with turpentine and 4 hours later bled 20 ml. per kilogram body weight by heart puncture. Two animals each were killed on the second and fifth days after bleeding, when the ratios of spleen to peripheral blood reticulocyte concentrations were found to average 3.3 and 2.1, respectively. Four rats that had received intraperitoneal methylcellulose for 10 weeks and showed marked splenomegaly, moderate anemia, and reticulocytosis were given turpentine intramuscularly and were killed 3 days later. In these animals the ratio of spleen to peripheral blood reticulocyte levels averaged 2.4, and was thus comparable to that of bled animals with turpentine abscesses. Reticulocyte levels in the lungs and livers were only slightly higher than that of the peripheral blood.

Estimations of the percentage of total circulating reticulocytes sequestered in the spleen in the various groups of animals studied were obtained by dividing

the product of the spleen hemoglobin content and spleen reticulocyte percentage by the product of the total circulating hemoglobin and peripheral blood reticulocyte percentage (Table III).

DISCUSSION

The tendency of rabbit reticulocytes to adhere to each other, to leukocytes, and to foreign surfaces such as glass, was described in 1922 by Key.[17] Although this sticky property of immature red cells when suspended in plasma or saline was found here to be striking in rabbits, it was distinctly less so in man and in

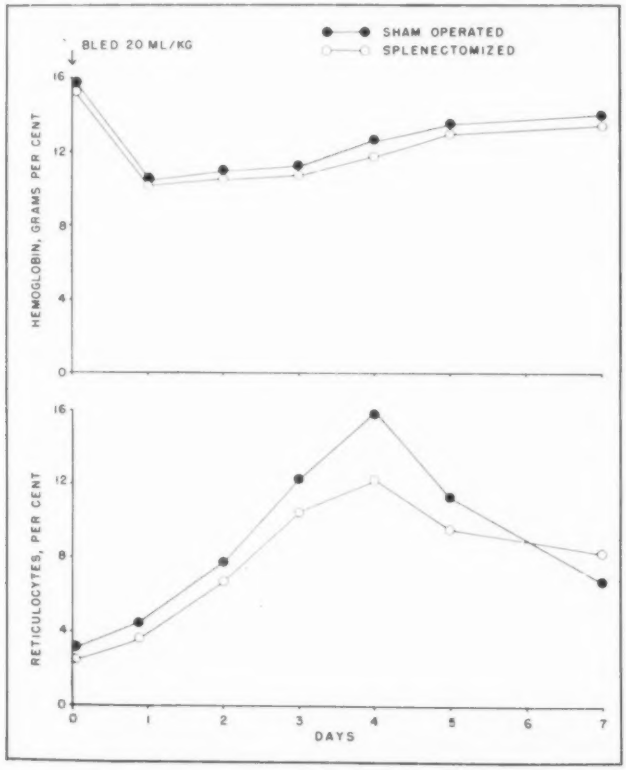

Fig. 5.—Comparison of hematologic responses to bleeding in splenectomized and sham-operated nonsplenectomized rats. Each point represents an average of 6 animals. There is no significant difference in the 2 groups, indicating that the normal spleen has no effect upon the acute erythropoietic response in the rat.

rats. Similarly, judging by the behavior of red cells in PVP, a rouleaux producing macromolecule, species differences exist in the agglutinability of mature red cells. In respect to both mature and immature red cells, these cells may be ranked by species in terms of agglutinability as follows: rabbit > man > rat.

The fact that the immature red cells of all 3 species tested are selectively clumped by PVP, even in man and rat, wherein clumping seldom occurs spontaneously or after centrifugation, indicates a property of the immature cell surface similar to that of cells coated with antibody, for in man PVP (K-44)

TABLE III. DISTRIBUTION OF RAT RETICULOCYTES BETWEEN SPLEEN AND PERIPHERAL BLOOD

SUBJECTS	BODY WEIGHT (GM.)	PERIPHERAL BLOOD				SPLEEN			RATIO, CONC. RETICULOCYTES SPLEEN:BLOOD	TOTAL CIRCULATING RETICULOCYTES IN SPLEEN (%)
		HGB. CONC. (GM. %)	ESTIMATED BLOOD VOL. (ML.*)	TOTAL CIRC. HGB. (MG.)	RETICULOCYTES (%)	WEIGHT (GM.)	HGB. CONT. (MG.)	RETICULOCYTES (%)		
Normal (16)	274	17.1	15.1	2,582	2.41	0.91	18.9	3.78	1.6	1.2
Bled normals (9)	250	10.3	13.8	1,421	7.70	0.82	17.5	13.60	1.8	2.2
Methylcellulose (9)	333	7.9	18.3	1,446	16.30	13.03	416	17.50	1.1	30.9
Bled + turpentine abscess (4)	260	8.5	14.3	1,216	9.80	0.95	18.0	24.60	2.5	3.7
Methylcellulose + turpentine abscess (4)	317	10.9	17.4	1,897	4.45	10.75	414	10.80	2.4	53.0

*The average normal blood volume of this strain of rat which weighs about 300 grams was found to be 5.5 per cent of body weight as determined by the Cr^{51}-labeled red cell method. This figure probably does not apply accurately to the abnormal animals studied.

has been found to agglutinate protein-coated red cells but not red cells abnormal in other ways.[10] More direct evidence that the surfaces of immature red cells contain protein is provided by the observation that the reticulocytes and normoblasts of all 3 species are selectively agglutinable by Coombs serum. The agglutinability of reticulocytes in Coombs serum was a function of the apparent morphologic youth of these cells, and, thus, was usually most striking in blood obtained during the response to severe anoxic stimulation. The fact that immature red cells are agglutinated by Coombs serum may explain the occasional published reports of a positive Coombs test in patients with nonimmunologic hemolytic anemias.[18–20] At least with respect to the species studied here (rabbit), this phenomenon appears to explain the report,[21] both confirmed[22] and denied,[23] that a positive Coombs test is demonstrable with the blood of animals previously injected with phenylhydrazine. The time sequence in the appearance of red cell agglutinability by Coombs serum in dogs given phenylhydrazine, as reported by Muirhead and associates,[21] is generally consistent with this supposition.

In lead-poisoned dogs and man Sutherland and Eisentraut[24] found that the red cells at the top of centrifuged blood specimens were often agglutinated by Coombs serum. Since reticulocytes as well as stippled red cells tend to be concentrated in the top layers of centrifuged red cells, the authors offered as one possible explanation the suggestion that reticulocytes may retain free globulin on the cell membrane, an explanation supported by this report.

The studies with Coombs sera which had been previously incubated with certain protein fractions indicate that the agglutinability of immature red cells is not due to a gamma globulin or albumin component in the cell membrane. Transferrin, on the other hand, consistently inhibited the agglutinating effect of Coombs serum on reticulocytes. This observation suggests that transferrin may actually become attached to the membrane of immature red cells, and remain attached despite washing of the cells in saline. Such an interpretation may explain previously reported evidence[4] that the iron bound to transferrin passes directly to the immature red cell without existing in a free form. This observation may provide evidence of a transport protein becoming attached to a cell specifically requiring the metabolite transported; there is indirect evidence that such an attachment occurs between the gastric intrinsic factor and the intestinal epithelium[25] and between insulin and muscle.[26] Theoretically it is conceivable that cellular agglutinability caused by the attachment of transferrin to immature red cells may be most striking in diseases such as lead poisoning which involve a pile-up of iron on the immature red cell membrane.[4, 27] Since the agglutination of reticulocytes by Coombs serum was not always completely blocked by transferrin, it is probable that other proteins are also present on the cell surface. There is also an equally tenable alternative possibility that the protein of the reticulocyte surface may resemble transferrin immunologically, and, indeed, chemically,[4] and yet be a different protein which forms a structural component of the immature cell membrane.

The fact that the Coombs test is of itself an immunologic procedure has caused some confusion as to its interpretation. This difficulty has been aggravated by the custom of referring to Coombs serum as an antiglobulin serum,

although it reacts with most, if not all, human serum proteins, regardless of whether whole human serum, or a fraction thereof, was used in sensitizing the donor animal. Thus the agglutination of washed cells by Coombs serum is not evidence of an immunologic phenomenon, but is only evidence of the presence of protein. The additional finding that the agglutinability of cells in Coombs serum is blocked by previous incubation with gamma globulin increases the likelihood that an antibody is attached to the cell, but this is still not specific evidence. Although the agglutination of red cells by Coombs serum is often taken as prima facie evidence of the presence on the red cell of an antibody directed specifically against the cell, the incorrectness of this assumption has been amply demonstrated. Thus, agglutinability in Coombs serum develops in red cells coated by an immunologic mechanism with drug-antibody complexes[28] and by a nonimmunologic mechanism with metalloprotein complexes.[29] The agglutinability of immature red cells must be regarded as a nonimmunologic phenomenon. The propensity of immature cells to aggregate (in Coombs serum, in PVP, and even to a slight extent in plasma) is probably no accidental quality. The tendency of like cells to aggregate may be considered a primitive feature of tissue organization[30] and may also be critical to the mechanism which controls the release of cells from the bone marrow. Conversely, the failure of immature cells to aggregate properly has been related to neoplastic invasiveness.[31] Conceivably a deficiency of this sort may account for the apparent invasiveness of the immature red cells of patients with acute erythremic myelosis (DiGuglielmo's syndrome).

The concentration of reticulocytes in the spleen, but not in the lungs or liver, of the rat was found to exceed significantly that in the peripheral blood. The absence of nucleated precursors in the spleen indicated that this was not the result of splenic erythropoiesis. The present finding confirms recently published observations by Berendes[32] who found the reticulocyte levels of blood in the spleen to exceed that of peripheral blood in human and animal subjects. Calculations based upon measurements of the amount of blood and the concentration of reticulocytes in rat spleens indicate that in the normal animal less than 2 per cent of the circulating reticulocytes are present in the spleen and that approximately one half of these are merely "passing through" that organ. After bleeding normal rats, the splenic trapping of reticulocytes increased somewhat, particularly in the first few days, when the ratio of reticulocyte concentration in the spleen to that in peripheral blood reached 2.4. The studies of the kinetics of injected Fe^{59} and of the distribution of Fe^{59}-labeled reticulocytes obtained from bled splenectomized rats and injected into normal rats indicate once again that only a small fraction (approximately 2 per cent) of immature red cells are trapped, probably transiently, in the normal spleen. That the fraction of immature red cells trapped by the normal rat spleen is very small is also attested by the response of splenectomized rats to bleeding. Except for the greater numbers of normoblasts in the peripheral blood from 2 to 4 days after the bleeding, these animals responded normally to blood loss. This corroborates studies on splenectomized dogs given phenylhydrazine.[33]

Splenic enlargement in rats caused by repeated intraperitoneal injections of methylcellulose produces hemolytic anemia and pancytopenia[34] associated with an increased vascularity, slowed circulation,[9] and increased sequestration of normal[6] and of abnormal[34] red cells within the spleen. These findings indicate that methylcellulose splenomegaly is analogous in many respects to many forms of hypersplenia in man primarily due to splenomegaly, such as congestive splenomegaly, the splenomegaly of certain acute infections,[35] and splenomegaly associated with some patients with leukemia,[36] agammaglobulinemia,[37] and Gaucher's disease.[38] Although the spleens of rats injected with methylcellulose studied here contained a large fraction (almost $\frac{1}{3}$) of the total red cell mass, there was no evidence of selective trapping of reticulocytes. Accordingly, it must be concluded that these congested spleens have either lost the ability to selectively trap reticulocytes, or, more likely, that the trapping process selects the old as well as the very young red cells. In contrast, the very slightly enlarged spleens of rats with turpentine abscesses showed a heightened trapping of reticulocytes, with a spleen to peripheral blood ratio of reticulocyte concentrations of 2.5. The spleens of rats afflicted both with turpentine abscesses and with methylcellulose splenomegaly manifested both selective reticulocyte trapping and red cell congestion. As a result, in these animals over one half (53 per cent) of the circulating reticulocytes were within the spleen at any given time (Table III). Thus, whereas normally the blood reticulocyte levels are affected very little by the presence of the spleen, in individuals with certain splenomegalic states* blood reticulocyte levels may be considerably depressed. These observations suggest that the apparent suppression of erythropoiesis, as judged by reticulocyte levels, in certain patients with splenomegaly is due to the selective filtration of reticulocytes rather than to a postulated hypersplenia with humoral inhibition of the bone marrow.

As distinguished from nonselective trapping of abnormal red cells throughout the reticuloendothelial system, such as occurs with red cells altered by storage[39] or by "complete" antibodies,[40] selective trapping of red cells in the spleen alone is observed in 2 general types of red cell alteration: (1) increased cell thickness, as in hereditary spherocytosis or with normal red cells made spheroidal by incubation with lecithin[39] or by heating[41] and (2) increased agglutinability as produced by incomplete antibodies[40] and certain metallic cations.[29] Both phenomena appear to promote sequestration by increasing the retention by physical filters of red cells under low perfusion pressures.[6] Immature red cells, being both bigger and more agglutinable than mature red cells, are thus doubly vulnerable to splenic sequestration. Studies in vitro of red cell filterability through Millipore† filters indicate that reticulocytes can be separated from mature red cells by simple filtration under low pressure and that, as with antibody-coated mature cells, this selective retention is greatly augmented by agglutination enhancing substances such as PVP and fibrinogen.[6]

*Presumably selective splenic trapping of reticulocytes could potentially occur in individuals with nonhemolytic diseases with splenomegaly, or with hemolytic processes acting independently of red cell age, but not in individuals with hemolytic diseases primarily affecting older red cells.

†Millipore Filter Corp., Bedford, Mass.

Thus in terms of their agglutinability and retention by filters in vitro and their susceptibility to splenic trapping in vivo, immature red cells and antibody-coated red cells behave similarly. The concept that reticulocytes may resemble malaria-parasitized red cells in that they are sticky and become trapped in organs with slow circulations was postulated in 1927 by Seyfarth.[42]

These studies do not reveal whether immature red cells trapped in the spleen are destroyed there or whether they simply mature there and then return to the circulation. The possibility that selective destruction of reticulocytes may account in part for the "early peak" of labelled fecal stercobilin after the administration of labelled glycine to normal subjects[43] has been mentioned by Watson.[44] Although this may be true in abnormal states, these studies in rats indicate that normally the amount of labelled pigment released if all the reticulocytes trapped in the spleen were destroyed probably would not be appreciable. The possibility that immature red cells may complete their maturation in the spleen and then be released is reasonable in terms of what is known of the behavior both of the reticulocyte and of the spleen. First, the reticulocyte is able to undergo morphologic maturation during stasis under otherwise physiologic conditions in vitro,[15] and in doing so, as shown here, the "ripened" reticulocyte loses its agglutinability. Second, the spleen has been observed in man to retain siderocytes[45] and lecithinized spherocytes[39] and then to return these cells to the circulation after correction of the red cell defects.* Similarly, mature, nucleated avian red cells injected into guinea pigs have been reported to extrude their nuclei while in the guinea pig spleens without undergoing apparent destruction.[47] Thus, the spleen may provide the function of ridding, or "pitting," red cells of inclusions and, in a broader sense, of modifying defective or underdeveloped cells, as suggested by Crosby.[45] It is reasonable to suppose that the sinusoids of the spleen, which morphologically are nearly identical to those of the bone marrow,[48] may thereby provide a secondary finishing station in the circulation to compensate for slight inefficiencies in the mechanisms which control the release of newly formed red cells into the circulation from the bone marrow.[49] Indeed, factors such as cellular agglutinability which may normally control the physiologic release of immature red cells from the marrow sinusoids, as was suggested by Key,[17] may by the same token lead to the retention of such cells in the splenic sinusoids. Since the agglutinability of immature red cells, at least in Coombs serum, may be due to the attachment of transferrin or the presence of a transferrin-like component in the cell membrane, it is conceivable that the diminishing physiologic need for this protein on the surface of immature red cells may be a controlling factor in their release from the bone marrow.

SUMMARY

The immature red cells of man, rabbit, and rat are selectively agglutinated by Coombs sera and by polyvinylpyrrolidone (PVP). In man at least part of

*The demonstration by Crosby[45] of the ability of the spleen to remove siderotic granules from red cells may provide an explanation, at least in part, of the excellent electron microscopic studies of Bessis and Breton-Gorius.[46] Although contrary to the interpretation of these workers, it is possible that the ferritin-rich granules observed were being removed from immature red cells by contingent histiocytes, rather than vice versa.

the protein coating of immature red cells is due to attached transferrin or to a structural protein immunologically similar to transferrin.

In rats the concentration of reticulocytes in blood in the spleen significantly exceeds that of peripheral blood. Normally less than 2 per cent of the total circulating reticulocytes are sequestered in the spleen. However, under certain experimental conditions over one half of the total reticulocyte population was concentrated in the spleen. It is suggested that selective splenic trapping of reticulocytes, rather than a splenic hormone, accounts for the apparent suppressive effect of the spleen on erythropoiesis in certain patients with splenomegaly.

The splenic trapping of immature red cells is believed to be caused by their being filtered from the perfusing blood by virtue of their increased agglutinability and greater size as compared to mature cells. It is postulated that the increased agglutinability which leads to splenic retention of immature cells is similarly involved in the physiologic retention of these cells in the bone marrow. It is conceivable than transferrin, and possibly, other transport proteins are involved in a device which is geared to cellular maturity for regulating the release of newly formed red cells from the marrow sinusoids. A function of the spleen sinusoids may be to temporarily retain cells which were released into the circulation prematurely until their maturation is sufficiently complete.

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PAPER ELECTROPHORESIS AS A METHOD OF EVALUATING THE EFFECT OF LONG-TERM ESTROGEN THERAPY ON SERUM LIPOPROTEINS IN MAN*

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AMPLE evidence has accumulated to show that most of the serum lipids are bound with globulins as lipoprotein complexes. Ultracentrifugal, chemical (solubility), and electrophoretic fractionation all show the bulk of the serum cholesterol and phospholipids to be carried by the α- and β-lipoproteins.

It is also well established that the serum α- and β-lipoproteins are quantitatively different in men and in women, and also in groups of atherosclerotic patients as compared with age- and sex-matched clinically normal persons.[3, 11, 12, 16, 18] In normal men under 35 years of age 25 to 35 per cent of the lipoproteins are found in the α-fraction and 52 to 75 per cent in the β-fraction.[1, 2, 7, 17] In atherosclerotic patients with clinically manifest coronary disease, on the other hand, the α-fraction decreases to 8 to 14 per cent whereas the β-fraction contains 85 to 91 per cent of the lipoproteins.[4, 15] There appears to be agreement on this point regardless of the method employed for lipid analysis, viz., paper electrophoresis with lipid staining[11, 20] or cholesterol determinations,[15] Cohn fractionation,[4] and ultracentrifugation.[11] Individuals with previous myocardial infarction have been chosen by various workers to be representative of the atherosclerotic group.[6, 15, 18] This is particularly valid in middle-aged patients who have suffered a myocardial infarction.

Several reports in the literature indicate that administration of female sex hormone preparations is capable of increasing the α-lipoprotein fraction in the serum of men with or without changes in the β-lipoprotein fraction, thus producing a female pattern of serum lipoproteins.[5, 8, 14, 18]

The present study was directed to the electrophoretic determination of the α- and β-lipoprotein fractions in a group of patients with proved myocardial infarction receiving either placebo or mixed conjugated equine estrogens

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*This is one of the reports resulting from the long-term study begun in 1952 in order to evaluate the therapeutic utility of estrogen in middle-aged men after myocardial infarction. The ultracentrifugal data used here were obtained for the over-all study by Drs. Lena Lewis and Irvine Page of the Cleveland Clinic. This material will be published separately later in extenso. Two interim reports have been published up to the present time on the over-all study.[18, 19]

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(Premarin).* The aim of the study was to determine (1) whether electrophoretic and ultracentrifugal results were comparable, and (2) whether this method could serve as a sensitive indicator for adherence to the prescribed dose schedule.

MATERIAL AND METHODS

Serum samples from 46 male patients were used. All patients were below the age of 50 when they joined the project; they were subsequently followed for from 1 to 5 years. All had suffered a proved myocardial infarction. Roughly one half the group was composed of controls who received placebo medication. The remainder received daily estrogen. A few of these received 10 mg. every other day for a short period of time, and one each was maintained on 2.5 and 4 mg. daily, respectively. Otherwise, all patients in the treated group received 10 mg. of Premarin daily. The patients were seen every 3 months, at which time fasting blood was drawn for the various determinations. The patients ate a general diet with no special emphasis on the fat intake.

Sera from all patients included in this report were sent to Drs. L. Lewis and I. Page at the Cleveland Clinic for ultracentrifugal lipid analysis. The initial electrophoresis was performed on all the serum samples within 1 to 3 days after the blood was drawn. Subsequent additional runs were made at intervals varying from 3 to 7 days. At least 2 runs were performed on each serum sample.

Electrophoresis was carried out in a plastic cell similar to that used by McDonald,[13] with a capacity of 6 strips of 1 by 15 inches Whatman No. 1 filter paper; the horizontal open strip method was utilized. Barbital buffer, pH 8.6, was prepared from Spinco B-1 buffer crystals. Current was applied to the buffer solution via calomel cells and agar salt bridges. The power supply was a Heathkit model PS-3, with a maximum output of 500 volts. Staining of the dried strips was performed according to the method of Swahn[20] utilizing a solution of Sudan black B in 50 per cent alcohol. The dried strips were scanned at intervals of 1 mm. with a strip attachment in the Beckman model B spectrophotometer at a wave length of 590 mμ. The optical densities of the lipoprotein peaks were plotted on graph paper and the area under each peak was measured with a planimeter. The results were expressed in square millimeters of dyed area.

The reproducibility of this method was tested in two ways: (1) 6 strips were run simultaneously using the serum from a single patient, and (2) a single serum sample was run repeatedly over a period of days to weeks. Both of these procedures were applied to a number of sera. The first method showed satisfactory reproducibility if strips which showed obvious technical faults were discarded. The second method showed a greater degree of variability. It was felt that storage of the sera caused some of the latter variability and consequently serum samples were always run within 1 week after the blood had been drawn. In addition, duplicate runs were made on each sample and the mean of these was used as the final result.

RESULTS

Originally it was intended that a quantitative method be utilized based on the work of Swahn.[20] The determination of total lipids was attempted using a triolein standard. We were unable to develop the degree of accuracy described by Swahn and, therefore, could not arrive at reliable quantitative results. Because the ratio between the dyed areas of α- and β-lipoprotein has been shown to remain constant in spite of alterations in the dye uptake of the individual fractions,[10] it was decided to express our results in terms of this ratio, the α:β lipoprotein ratio.

The administration of estrogen caused a definite increase in the α:β lipoprotein ratio in those patients in whom control and treatment blood samples

*Supplied by Dr. John B. Jewell of Ayerst Laboratories, New York City, N. Y.

were available. Most of the patients in the treated group, unfortunately, were already under therapy when this study was started. Only 4 such patients were available. Two were new patients and 2 were previously on therapy but subsequently discontinued medication. The results are shown in Table I. It can be seen that the alteration of the α:β lipoprotein ratio occurred within at least 3 to 4 weeks after therapy was begun, and disappeared within the same period

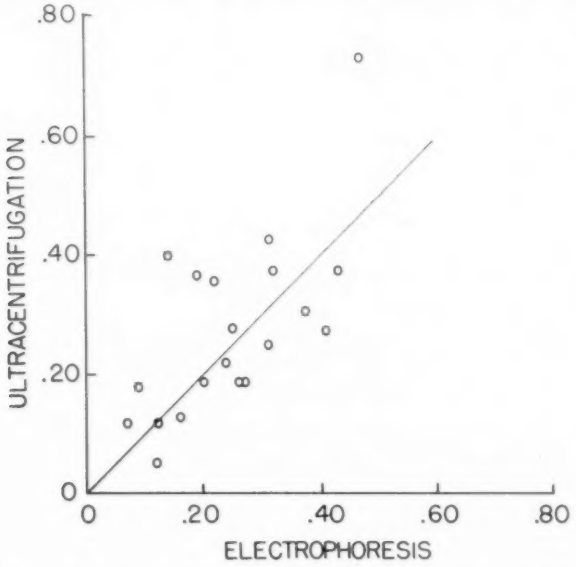

Fig. 1.—Comparison of electrophoretic and ultracentrifugal α:β lipoprotein ratios in 20 placebo treated patients.

after it was discontinued. Comparison of the α:β lipoprotein ratios with cholesterol values and cholesterol:phospholipid ratios demonstrates that the lipoprotein ratio is the only 1 of 3 determinations that shows a significant change in quantity and direction in this relatively short time interval. With longer periods of observation of the entire group of patients, cholesterol:phospholipid ratios decreased consistently as expected when α-lipoproteins are elevated without elevation of β-lipoproteins.

This is admittedly too small a sample for any conclusions to be drawn as to the reliability and sensitivity of the lipoprotein method. However, in all patients used in this study pretreatment α:β lipoprotein ratio determinations by ultracentrifugation were available. Also available were parallel determinations of ultracentrifugal and electrophoretic data in the placebo treated patients. There is a fairly close correlation between the results with the two methods (Fig. 1). The α:β lipoprotein ratio was significantly increased in the estrogen treated patients when the results (with each of the two methods) are compared with the pretreatment data obtained by ultracentrifugation. A comparison of α:β lipoprotein ratios in this study, therefore, indicates that their determination by electrophoresis is a sensitive indicator of estrogen induced lipoprotein shifts in individual patients.

It was hoped that a comparison of the $\alpha:\beta$ lipoprotein ratios of treated and untreated patients would demonstrate a clear point of separation, so that the presence or absence of therapeutic effect could be easily determined. A scattergram of the mean electrophoretic and ultracentrifugal results of the 46 patients, 20 control and 26 treated (Fig. 2), shows that there is an overlap between the two groups with each of the 2 methods. Further, the ultracentrifugal $\alpha:\beta$ lipoprotein ratios in estrogen treated patients are consistently higher than the ratios obtained by electrophoresis. Separation of patients on estrogen from those not on therapy is possible from the use of the over-all means of each group. Values falling below the over-all mean of the untreated group (0.24 for electrophoresis and 0.28 for ultracentrifuge) and above the over-all mean of the treated group (0.48 for electrophoresis and 0.64 for

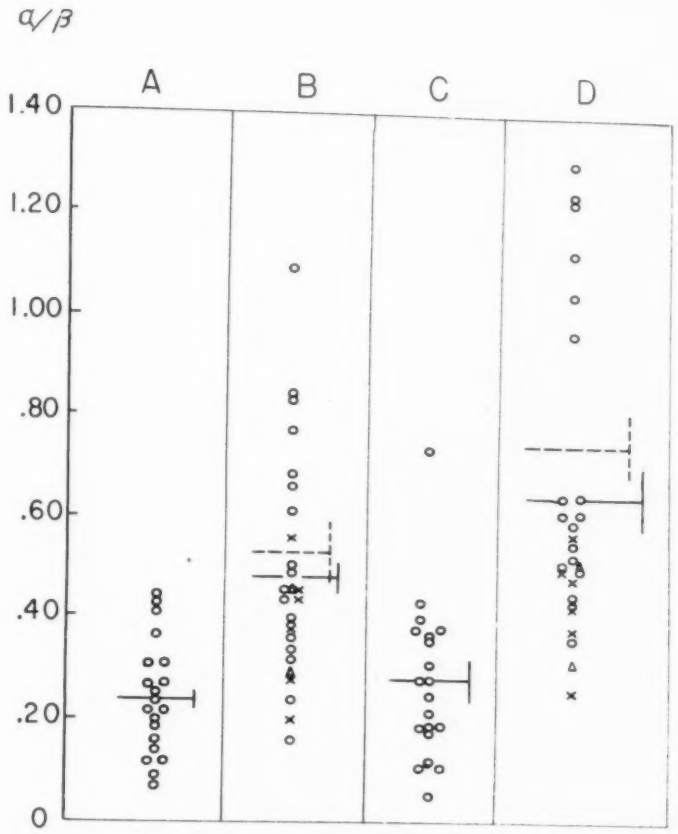

Fig. 2.—Scattergram of electrophoretic and ultracentrifugal $\alpha:\beta$ lipoprotein ratios in 20 placebo and 26 estrogen treated patients. *Column A.* Electrophoresis—placebo patients. *Column B.* Electrophoresis—estrogen patients. *Column C.* Ultracentrifuge—placebo patients. *Column D.* Ultracentrifuge—estrogen patients.

×, Patients with irregular or questionable drug adherence to 10 mg. of Premarin.

○, Patients with 2.5 or 4 mg. of Premarin daily. The solid horizontal lines are the over-all means of each group; the vertical lines on the right, the standard error of the mean. The broken lines represent the same values when patients × and ○ are excluded from the calculation.

ultracentrifuge) are usually indicative that a patient is on placebo and estrogen, respectively. The values between the over-all means are not definitive in this respect.

In any one patient the mean α:β lipoprotein ratio as determined by electrophoresis showed fairly good correlation between samples drawn at 1 to 3 month intervals (Fig. 3).

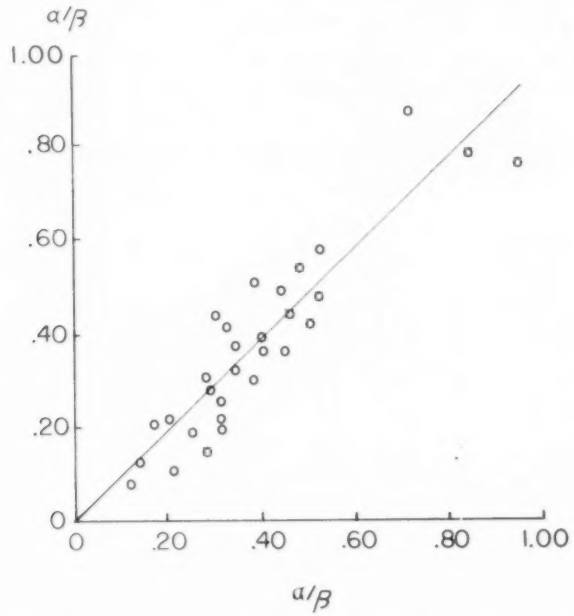

Fig. 3.—Each point represents the simultaneous plotting of the mean α:β lipoprotein ratios of 2 different serum samples obtained from an individual patient at 1 to 3 month intervals.

Calculation of the α:β lipoprotein ratios from Gottfried's[9] results for clinically healthy men, 40 to 49 years of age, gave a mean value of 0.49, which is close to the value (0.48) of the treated patients in our series. By comparison, our control patients with atherosclerosis had a mean ratio of 0.29.

DISCUSSION

Patients with atherosclerosis, specifically middle-aged men with a previous myocardial infarction, are known to have altered blood lipids when compared to clinically normal men of the same age group. The α:β lipoprotein ratio most reliably demonstrates the alteration in such instances. Estrogen therapy can increase this ratio to values seen in young women. This process is readily reversible in either direction and the change can occur in as short a time as 3 to 4 weeks.

In comparing an estrogen treated and a control group of postmyocardial infarction patients in the present study, in which there was no absolute separation of the α:β ratios, an obvious overlap zone was found to exist. This was

true for both the electrophoretic and ultracentrifugal results. Use of the over-all means of the treated and untreated groups, however, did provide a method of clear separation between treated and untreated patients.

A comparison of the control and therapeutic α:β lipoprotein ratios of individuals receiving estrogens demonstrated an obvious elevation while they were on therapy. Upon comparing the α:β lipoprotein ratios in patients on treatment a short time with others on treatment for several years, no difference was found in the degree of elevation. Individual patients showed surprising constancy of their α:β lipoprotein ratios.

It is concluded from these data that paper electrophoresis with lipid staining is a reliable method for the evaluation of the ability of estrogens to elevate the α:β lipoprotein ratio in individual patients. It is more useful in individual cases than for the entire group. It may, therefore, also be helpful to determine the dose of hormone required to elevate α-lipoprotein in a given individual. Furthermore, it can serve in checking drug adherence—a serious problem in long-term studies.

TABLE I. COMPARISON OF MEAN α:β LIPOPROTEIN (α:β) RATIOS, SERUM CHOLESTEROL, AND CHOLESTEROL:PHOSPHOLIPID (C:P) RATIOS OF PATIENTS WHILE ON AND OFF ESTROGEN THERAPY

PATIENT NO.	DATE	RELATION TO ESTROGEN THERAPY	α:β RATIO	CHOLESTEROL (MG. %)	C:P RATIO
1	2/ 7/57	Before	0.19	383	1.26
	3/ 7/57	On 1 month	0.33	308	1.16
2	12/11/56	Before	0.25	355	0.92
	1/ 8/57	On 1 month	0.45	304	0.90
	2/ 5/57	On 2 months	0.38	405	0.97
	3/ 5/57	On 3 months	0.46	315	0.93
3	1/10/57	On for some time	0.43	210	0.91
	4/ 4/57	Off 3 months	0.15	186	0.93
4	12/11/56	Off 3 weeks	0.18	268	0.90
	3/ 5/57	On 3 months	0.48	258	0.92

SUMMARY

1. The α:β lipoprotein ratio of serum from individuals with atherosclerosis as determined by electrophoresis was elevated after the oral administration of estrogen (Premarin). This elevation persisted even after several years of therapy.

2. Upon discontinuation of hormone therapy the α:β lipoprotein ratio fell markedly in 3 to 4 weeks. With institution of therapy the ratio became elevated within a similar period of time.

3. There was considerable overlap of α:β lipoprotein ratios between the treated and control groups whether the electrophoretic or ultracentrifugal method of lipid analysis was used. However, the mean α:β lipoprotein ratios of these two groups were significantly different, and values outside the region between the over-all means had significance for individual patients.

4. The electrophoretic method described serves as a useful tool in assessing drug-adherence in estrogen treated patients.

Drs. P. de Rabago, D. Berkson, S. Kimelblot, J. D. Voltmann, J. Frankel, and Sister Mary Alma contributed to the study at various stages of its development. Mrs. D. Century, Mrs. M. Vankinscott, and Miss M. Glazwand gave valuable technical assistance.

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THE EFFECT OF EXERCISE ON PULMONARY BLOOD FLOW AND ON LEFT-TO-RIGHT SHUNT IN PATIENTS WITH VENTRICULAR SEPTAL DEFECTS AND NORMAL PULMONARY ARTERIAL PRESSURES

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MANY patients with ventricular septal defects and left-to-right shunts do well clinically for many years. Some of them live a normal span of years and are able to do appreciable amounts of physical work with little difficulty. An examination of the characteristics of the pulmonary circulation and its response to certain stresses such as exercise as well as a knowledge of the factors influencing the volume of blood diverted from normal channels through the defect might explain some of the clinical observations in such patients. The pulmonary arterial pressure in many patients with ventricular septal defects is normal or only moderately elevated. This can be explained in part by the fact that the pulmonary circulation has a large functional reserve and is well able to tolerate large increases in blood flow without changes in the pulmonary vasculature for appreciable lengths of time. Furthermore, the right ventricle is essentially a "volume pump" and is well adapted for moving relatively large amounts of blood against the low resistance of the normal pulmonary vascular tree. These functional characteristics of the right ventricle are in contrast with those of the left ventricle which is much better adapted for pumping against a relatively high resistance. The function of the right ventricle is not compromised severely by a volume overload if the pulmonary vasculature is relatively normal. Considering the capacity of the right ventricle and of the pulmonary circulation, it is not surprising that patients with ventricular septal defects and left-to-right shunts may tolerate increased pulmonary flow for long periods before vascular changes which produce increased resistance to flow lead to pulmonary hypertension with its accompanying deleterious effects on the right ventricle. These remarks obviously do not apply to situations in which the ventricular septal defect is so large that the right ventricle and the pulmonary vessels are subjected to the stress of high pressures transmitted from the left ventricle and to huge volume overloads very early in life.

The relatively large pressure gradients across ventricular septal defects in patients without pulmonary hypertension might lead one to expect an increase in the volume of the left-to-right shunt out of proportion to increases in systemic

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flow during exercise. This might in turn compromise the efficiency of both ventricles in terms of energy expenditure for the useful work performed. The pressure-flow relationships in patients with septal defects at rest are quite well understood, but less information is available on the changes in left-to-right shunts during exercise. Swan and co-workers[1] have studied the effects of exercise on a group of patients with left-to-right shunts of various types. They showed that pulmonary blood flow at rest and during exercise was relatively fixed when pulmonary hypertension existed. Increased resistance to flow in the pulmonary arterial tree appeared to be more important in limiting flow than the size of the defect or other factors. Swan's study also showed that his patients with left-to-right shunts not associated with pulmonary hypertension, including two who had ventricular septal defects, responded to exercise with increased pulmonary blood flows but that the relative shunts from left to right tended to decrease. Our study is also an attempt to measure the changes in systemic and pulmonary blood flow during exercise in patients with isolated ventricular septal defects and normal pulmonary artery pressures.

MATERIALS AND METHODS

The patients in this series had typical ventricular septal defects with characteristic physical signs. None of the patients were seriously incapacitated as a result of the defect and all were in relatively good health. The clinical diagnoses were verified by cardiac catheterization at the time of the cardiac output studies. A very small atrial septal defect not detected at cardiac catheterization was found in Patient 9 during cardiotomy for repair of her ventricular defect. The atrial defect probably was not associated with a significant shunt. All but one of the patients have had cardiotomies and repair of their defects. The pulmonary artery pressures did not exceed 40 mm. Hg systolic in any of the patients. Right-to-left shunts were not present. All cardiac output studies were done with the patients in a fasting condition. Several patients received 0.1 Gm. of Nembutal orally approximately 1 hour before the procedure but no additional sedation. One cardiac catheter was placed with its tip lying in the proximal portion of the right pulmonary artery. A second catheter, usually a No. 5 or 6 French, was introduced into the basilic vein through the same cut-down or through another tributary of the basilic vein. The second catheter was advanced until its tip was lying free within the right atrium. One of the brachial arteries was cannulated with a Cournand needle. Expired air was collected in a Tissot spirometer and simultaneous blood samples were obtained from the brachial artery, pulmonary artery, and right atrium during the air collection. All blood gas analyses were done by the Van Slyke method. After the resting cardiac outputs, leg exercise was begun, using a bicycle ergometer, and was continued for periods as indicated in Table I. The patients remained in the supine position. Gas collections and blood samples for the exercise outputs were obtained near the end of the period of exercise. An attempt was made to have the patient exercise at as constant a rate as possible. Others have shown that oxygen uptake and the A-V oxygen difference reach a steady states very quickly under such circumstances.[2] The spirometer was rinsed with expired air during exercise before the final collection for the determination of oxygen consumption. All values for oxygen consumption and blood oxygen content in Table I are given in terms of standard conditions (0° C. and 760 mm. Hg dry). All pressure measurements were made immediately after sampling; strain gauges were used. Mean pressures were obtained by electrical integration. All pressures associated with exercise outputs were made while the patient was exercising rather than immediately afterward. Peripheral resistances were calculated in terms of dynes · sec · cm.$^{-5}$. Pulmonary capillary or left atrial pressures were not measured; therefore, the values for the pulmonary

circulation do not represent arteriolar resistance but total resistance. The left-to-right shunts have been given as liters of blood flow per minute and as a percentage of total pulmonary blood flow.

Determinations of systemic blood flow under these circumstances are not entirely satisfactory because of the difficulties inherent in obtaining representative samples of mixed venous blood. Tricuspid valve lesions which produce insufficiency and the escape of shunted arterialized blood into the atrium were not present in these patients. Neither was there evidence of left ventricular–right atrial shunts. The one patient with a very small atrial septal defect did not have a detectable left-to-right shunt at the atrial level. Some variation in oxygen content of the atrial samples would be expected to occur on the basis of incomplete mixing of blood of varying oxygen content from the cavae, although several authors have suggested that true mixed venous blood can be obtained from the right atrium if the catheter is placed properly.[3-5] Even though estimations of systemic blood flow may not be entirely accurate under these circumstances, the consistency of the data with respect to the calculation of the relative shunts do suggest a definite trend.

RESULTS

The pertinent data are given in Table I. The systemic arterial oxygen saturation stayed within normal limits during exercise in all patients, and although this does not absolutely rule out a small admixture of venous blood with the systemic arterial blood, a shunt from right to left seems unlikely in any case. The pulmonary artery oxygen content decreased in every patient during exercise. The fall in the pulmonary arterial oxygen content during exercise was not associated with a comparable decrease in the right atrial oxygen content in 4 patients (Patients 2, 6, 7, and 9). The calculated pulmonary blood flow increased in these 4 patients during exercise, but examination of the changes in oxygen content of the blood in the right atrium and pulmonary artery will show that the fraction of total pulmonary flow from the left-to-right shunt must have decreased. The greater fall in oxygen content in the pulmonary artery than in the right atrium during exercise is an indication of a larger portion of mixed venous blood in the output from the right ventricle. This increased contribution of mixed venous blood to the pulmonary flow also means that the right ventricle expends less energy in pumping shunted blood and that the effective work of the right ventricle increases.

The largest increase in the relative shunt from left to right expressed as a fraction of total pulmonary blood flow was only 11 per cent after exercise (Patient 1). With this exception, exercise either produced insignificant changes or was associated with a decrease in the fraction of blood diverted back to the pulmonary circuit through the ventricular defect. Equally interesting is the decrease in the absolute volume of the left-to-right shunt in 5 patients.

Pulmonary artery pressures generally change very little in normal people who exercise at levels used by the patients in this group.[6] The changes in pulmonary artery pressures during exercise in our patients with ventricular septal defects were not significantly different from the changes seen in normal people. The mean pulmonary artery pressure rose an average of only 3.6 mm. Hg. The changes in brachial artery pressure during exercise were also small, but the net effect of increased systemic flow with these pressures was a decrease in the calculated peripheral resistance for most patients. Decreased resistance to flow through the systemic arterial bed during exercise may be one factor which tends

TABLE I. HEMODYNAMIC DATA IN 10 PATIENTS WITH VENTRICULAR

PATIENT NO.	AGE	SEX	SURFACE AREA ($M.^2$)	EXERCISE (MIN.)	O_2 CONSUMPTION (ML./MIN.)	OXYGEN CONTENT			ARTERIAL SATURATION (%)
						BRACHIAL ARTERY	PULMONARY ARTERY (VOL. %)	RIGHT ATRIUM	
1	19	F	1.62	Rest	204	16.80	12.81	11.65	97
				9	631	17.01	12.02	9.49	97
2	19	M	1.87	Rest	291	19.28	16.20	12.67	98
				10	735	19.33	13.92	12.26	98
3	22	F	1.45	Rest	246	15.80	14.26	11.63	
				7	528	15.91	11.85	7.80	99*
4	24	M	1.92	Rest	237	18.88	15.71	15.13	100
				7	549	19.33	12.87	11.45	100
5	30	F	1.63	Rest	204	16.38	13.55	11.56	100
				4	540	16.78	12.34	9.44	100
6	15	F	1.53	Rest	209	18.02	14.94	12.44	99
				4	710	18.26	12.59	12.16	98
7	29	M	1.77	Rest	265	19.46	15.62	14.42	100
				10	688	19.18	13.65	13.29	96
8	9	M	0.97	Rest	222	14.58	12.39	10.49	99
				4	515	14.69	11.29	8.98	100
9	15	M	1.33	Rest	220	16.54	13.85	11.71	97
				7	535	17.16	11.79	10.27	99
10	11	F	1.28	Rest	240	15.12	11.86	10.96	99
				6	617	15.24	9.54	7.27	100

*Oximeter reading.

to limit the left-to-right shunt during exercise, but it may not be the only one. The size and location of the defect are undoubtedly important. It is possible that contraction of the septum during systole may decrease the area of the defect, particularly if the defect lies in the muscular septum, and, in this way, reduce the shunt during the ejection phase. Since left ventricular end diastolic pressure is normally higher than that of the right ventricle, there is also a possibility that some arterialized blood may flow across the defect from left to right during the diastolic period. If such a mechanism is operative to a significant extent, particularly in large defects, an increase in heart rate as a normal response to exercise would shorten the diastolic filling time and might limit this part of the left-to-right shunt.

SUMMARY

Simultaneous determinations of pulmonary and systemic blood flows were made during rest and exercise in ten patients with uncomplicated ventricular septal defects and normal pulmonary artery pressures. The usual response to exercise was an increase in pulmonary blood flow. The calculated volume of blood shunted from left to right decreased in some patients. More significant is the fact that the relative left-to-right shunt expressed in terms of total pulmonary blood flow showed either very small changes or actually fell during

SEPTAL DEFECTS NOT ASSOCIATED WITH PULMONARY HYPERTENSION

BLOOD FLOW		LEFT-TO-RIGHT SHUNT		PRESSURES				RESISTANCE	
				PULMONARY ARTERY		BRACHIAL ARTERY			
PULMONARY (L./MIN.)	SYSTEMIC (L./MIN.)	(L./MIN.)	% OF TOTAL PULMONARY FLOW	MM. Hg	INTEGRATED MEANS	MM. Hg	INTEGRATED MEANS	PULMONARY DYNES · SEC · CM.$^{-5}$	SYSTEMIC DYNES · SEC · CM.$^{-5}$
5.11	3.96	1.15	23	23/9	13	134/60	101	203	2,040
12.65	8.39	4.26	34	26/10	15	167/91	113	95	1,080
9.45	4.40	5.05	53	30/13	18	112/72	84	152	1,530
13.59	10.40	3.19	23	33/18	24	126/67	94	141	720
15.97	5.90	10.07	63	33/14	21	121/62	84	105	1,140
13.00	6.51	6.49	50	38/15	25	118/57	82	154	1,010
7.48	6.32	1.16	16	25/9	16	116/61	74	171	940
8.50	6.96	1.54	18	28/11	19	126/68	82	179	940
7.21	4.23	2.98	41	22/10	15			166	
12.16	7.36	4.80	39	20/9	14			92	
6.79	3.75	3.04	45	22/9	13			153	
12.52	11.64	0.88	7	31/15	20			128	
6.90	5.26	1.64	24	27/11	18	111/74	92	208	1,400
12.44	11.68	0.76	6	36/13	22	112/78	99	141	680
10.14	5.43	4.71	46	22/12	17	139/83	103	134	1,520
15.15	9.02	6.13	40	25/15	20	143/73	101	106	900
8.18	4.55	3.63	44	19/5	10	116/69	82	98	1,440
9.96	7.76	2.20	22	23/8	13	105/46	78	104	800
7.36	5.77	1.59	22	27/12	19	138/76	100	206	1,390
10.82	7.74	3.08	28	35/19	24	136/84	96	177	990

exercise. In the circumstances described, the heart and circulation are able to adapt to the stress of exercise without an excessive increase in the work of the right ventricle. A higher percentage of the total work of the right ventricle during exercise is useful work as a consequence of the decreased left-to-right shunt.

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BIOLOGIC PROPERTIES OF HUMAN LEUKEMIC AND TUMORAL RNA

III. The Effect of Different Media on the Cytopathogenicity in Tissue Culture

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TRANSMISSIBLE cytopathogenic changes in primary cultures of human amnion, produced by RNA from human leukemic and solid tumor cells, extracted with a fluorocarbon, were described previously.[1-3] Exponential increase of the cytopathogenic titer through serial passage was observed (Fig. 1). Frequent failure of a passage to produce cytopathogenic changes was usually interpreted as the result of the instability of the quasi-protein free RNA preparation; in many instances, however, after a blind passage, cytopathogenicity could be recovered and passed again. Stabilization of the RNA preparation with EDTA increased the incidence of cytopathogenic passages.[2, 3] Since stabilization of the RNA preparation did not seem to be the only factor involved in the production of the cytopathogenic changes, other variables were also studied. Already it was observed that an increase of the pH of the medium had a potentiating effect.[3] The results of using different tissue culture media and sera on the reproducibility of the cytopathogenic effect are presented in this article.

METHODS AND MATERIALS

Preparation of the Nucleic Acid.—While trying the method of deproteinization by high speed homogenization with a fluorocarbon[4] to uncover hypothetical viral agents from human neoplastic tissue, it was found that, if the deproteinization was carried, by repeated extractions, to the extent that protein reactions become negative and the absorption at 2,800 Å negligible, the supernatant fluid had an absorption spectrum characteristic of purines and pyrimidines (Fig. 4, *C*). Further characterization by the method of Hotchkiss[5] (Fig. 4, *D*) and quantitative determination by the orcinol and diphenylamine methods[6] and of the nitrogen:phosphorus ratio indicated the presence of RNA and DNA in a proportion of about 10:1 (RNA:DNA). It was later found that the biologic properties shown by the preparation could be abolished by ribonuclease but not by desoxyribonuclease.[2, 3]

In the technique of Gessler,[4] the virus containing tissues were previously homogenized in a buffer solution and the homogenate was emulsified with fluorocarbon. Since, after exhaustive deproteinization, a quasi-protein free nucleic acid was obtained, it was decided to eliminate the first step to permit denaturation and inactivation, by the fluorocarbon, of proteins and enzymes, as the are freed, and prevent these from acting upon water-soluble or dispersed substrates.

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Since the fluorocarbon is a nonpolar detergent, this method does not differ fundamentally from other methods of nucleic acid extraction[7] and has the advantage that nucleic acid, protein, and detergent become automatically separated.

Instrument.—Air turbines driven by 100 pounds of air pressure were adapted to operate with the Vir-Tis stainless steel shaft and blades. On stroboscopic observation the air motor* delivered speeds from 40,000 to 50,000 r.p.m. The air turbine "Jack"† speeds up to 70,000 r.p.m.‡

Because of the possible inactivating effect of iron ions on the nucleic acids,[8, 9] the shaft and blades, as well as all glassware, were washed in 10 per cent disodium Sequestrene, rinsed in deionized distilled water, silicone coated,§ and kept dry and sterile at −20° C. until used.

The Fluorocarbon.—1-fluoro-dichloro-2-dichlorofluoroethane‖ is a nonpolar, organic detergent of low boiling (47.7° C.) and freezing (−36.6° C.) points, high density (1.574 Gm. per milliliter at 20° C.), low surface tension (17.75 dynes per centimeter) and viscosity (0.704 centipoise at 20° C.). The solubility in water at 1 atmosphere is 0.028 per cent and its emulsion with water resolves easily and completely upon standing or at low-speed centrifugation. Its toxicity is reported as being of the order of that of CO_2.[4] Before use the fluorocarbon is distilled and kept at −20° C.

Materials.—Fifty to 100 ml. of peripheral blood and 2 to 5 ml. of bone marrow from the leukemic patient were collected in ACD solution or 3 and 10 per cent disodium ethylenediamine tetracetate (9 parts blood to 1 part anticoagulant). Differential counts in these samples varied from 4×10^7 to 2×10^{10} leukemic cells per cubic millimeter. Initially the leukocytes were separated by addition of fibrinogen, dextran and/or phytohemagglutinin.[10] Experience showed that direct homogenization of chilled whole leukemic blood and bone marrow with the fluorocarbon yielded the same or better results. Pieces of tumor tissue, cleaned of necrotic or hemorrhagic parts, weighing from 1.39 to 145 grams, were minced and suspended in 0.15M, pH 7.2 Sørensen phosphate or McIlwaine's citric acid buffer at 0° C. The water used was multiple distilled and deionized to 0.1 part per million of NaCl.

Control materials consisted of the fluorocarbon itself, 20 samples of blood and 3 of bone marrow from different nonleukemic individuals, whole human placenta, and 5- to 10-day-old human amnion cultures. A fluorocarbon-treated commercial preparation of RNA from bovine pancreas¶ was included and also served as a standard for spectrophotometric determinations of nucleic acid (Fig. 4, *C*).

To 1 part chilled blood or tumor suspension in buffer was added 1 part chilled fluorocarbon. The mixture was homogenized at 45,000 to 50,000 r.p.m. for 10 minutes or at 70,000 for 5 minutes with the homogenizing cup immersed in a bath of ice-acetone which maintains the temperature of the homogenate between 0° and 4° C. After centrifuging the homogenate for 10 minutes at 1,800 r.p.m. in a PR-2 International centrifuge (2° C.), the homogenate separates in 3 layers. The supernatant is pipetted with a chilled pipette into a chilled cylinder and the original volume of blood or of the tumor suspension is reconstituted with chilled buffer. Repeated homogenizations of the supernatant are carried until this becomes a clear transparent fluid and no precipitate appears between the aqueous phase and the fluorocarbon. The final homogenate is then filtered through chilled porcelain filters of 0.60 μ average pore size (FMB-52-03 Selas); the filtration is carried between 0° and 4° C. For sizing the particulate material and comparing with electronmicroscopic measurements,

*Model D-039, Rotor Tools, Cleveland, Ohio.
†Buckey Tool Corporation, Dayton, Ohio.
‡The adaption of Vir-Tis shafts to the air motors was made by the courtesy of J. Nowalanic, model shop supervisor, R.D.C.
§11007, Dow Corning Corp., Midland, Mich.
‖Genetron 113, Allied Chemical and Dye Corporation, New York, N. Y.
¶Nutritional Biochemicals Company, Cleveland, Ohio.

the fluids were passed through a gradient of membrane filters (Millipore) of porosities 100 ± 8, 50 ± 3, and 10 ± 1 mμ. For the purposes of phosphorus[11] and nitrogen[12] determinations and of electronmicroscopic examination, the preparations were dialyzed in cellophane (240 mμ average pore size, Visking) for 4 hours against distilled water at 2° C.

Electronmicroscopic studies were performed in an EM 100 Phillips instrument. A drop of the dialyzed supernatant fluid was deposited or sprayed on collodion or carbon substrates and shadowed with pure gold or gold-copper and gold-palladium alloys. The voltage acceleration was usually 100 kv. and the objective aperture 30 μ.

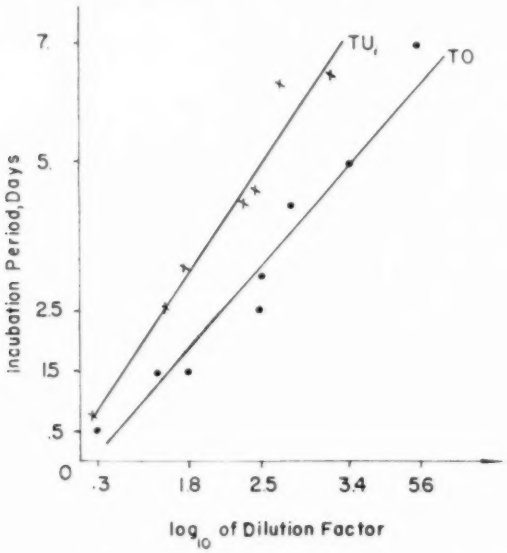

Fig. 1.—The cytopathogenic effect (CPE) was titered by the usual virologic dilution methods. In this case inverse proportionality is shown between dilutions and the time required to obtain CPE_{2+} (4+) in the same passage.

Primary cultures of human amnion from caesarean sections were prepared from cell suspensions obtained by trypsinization of the membranes. One-half to one million cells per milliliter of medium were seeded in roller tubes with 2 ml. of medium, pH 7.2. The incubation was stationary at 25 and at 37° C. Cultures were used as soon as solid monolayers were formed (Fig. 2, *A*).

The medium used in the former experiments, designed to detect cytopathogenicity of the fluorocarbon homogenate, was composed of 9 parts 199 Parker's synthetic solution and 1 part AB human serum. The serum was dialyzed against distilled water in continuous flow at 2° C., centrifuged, filtered through previously washed Seitz pads, divided in portions, and frozen at –20° C. Before use it was heated at 56° C. for 30 minutes. Samples of this serum were tested for toxicity on cultures at 0.2 ml. of serum per milliliter of complete medium. If no cellular changes occurred before controls inoculated with nontoxic serum started degenerating, the serum was considered nontoxic.

Passages.—The tubes to be harvested are frozen and the monolayer of cells is scraped off the glass with a Teflon policeman and suspended in the culture fluid. This suspension is homogenized with fluorocarbon and filtered in the manner described for blood. Cells grown on cover slips were studied in phase and vital stained or fixed and stained. Only a limited number of sources of materials were used. The variables introduced were the combination of different media and sera with cultures of varying growth characteristics.

These cultures were made from amnion membranes obtained at cesarean sections. They were started with 10^6 cells per milliliter, the fluid was changed 3 to 4 times, and the

cells were subcultured 2 to 3 times by trypsinization. They were inoculated when 7 to 20 days old. Human AB serum and calf serum* were dialyzed and inactivated as described. 199 Parker and NCTC 107 solutions† were used in 4 combinations with 10 per cent of the 2 sera.

The following were the sources of materials used in the present work:

$LK_{17}TC$: fluorocarbon extracted RNA from the first passage, in amnion culture, of RNA extracted from the leukemic cells of Case LK_{17}, an acute leukemia in an 8-year-old child.

$LK_{27}LN$: RNA from a group of lymph nodes biopsied from an adult patient with Hodgkin's paragranuloma.

$LK_{29}C$: RNA from a 10-day-old culture of the leukemic cells of Case LK_{29}, acute stem cell leukemia in an adult. This primary culture of human leukemic cells was started with 199 solution and 10 per cent of the patient's own leukemic serum maintained with 10 per cent of human cord serum, dialyzed, and inactivated.[2, 3] The culture was maintained for about 6 months with 10 passages, and was lost accidentally.

LK_{29}: RNA from the original circulating leukemic cells of the same patient.

LK_{28}: RNA from the leukemic lymphocytes of Case LK_{28}, chronic lymphatic leukemia in an adult.

LK_{32}: RNA from the circulating leukemic cells from Case LK_{32}, acute stem cell leukemia in an adult.

TU_{24}: RNA from a piece of a breast carcinoma obtained at a mastectomy from a woman.

NA: RNA from 6 tubes of a 10-day-old culture of human amnion grown in 199 plus 10 per cent of dialyzed, inactivated, AB human serum.

HeLa: RNA from 6 tubes of HeLa (Gey)* and subcultured in our laboratory with Ginsberg's maintenance fluid.

TABLE I. CYTOPATHOGENIC EFFECT OF LEUKEMIC RNA UNDER DIFFERENT MEDIA

		MEDIA						
		1		2	3	4		
		199 + 10% AB SERUM		199 + 10% CALF SERUM	NCTC 107 + 10% AB SERUM	NCTC 107 + 10% CALF SERUM		
INOCULA	RNA (μG/ML.)	CA88†	CA93†	CA87	CA93	CA90	NUMBER OF CONSECUTIVE PASSAGES	CPE TITER AFTER LAST PASSAGE: LOG_{10} OF DILUTION FACTOR
$LK_{17}TC_3$	0.79	–	–	–	–	+	3	3.1
$LK_{27}LN$	1.71	–	–	–	–	+	7	6.1
LK_{29}	0.16	–	–	–	–	–	0	0
LK_{29}	1.92	–	+	+	+	+	9	6.2
$LK_{29}C$	0.57	–	+	+	+	+	8	6
LK_{32}	0.73	–	–	–	+	+	2	1.6
J-111		–	–	+	–	+	8	5.4
NL	0.90	–	–	–	–	–	6	0
NHA	0.95	–	–	–	–	–	6	0
TU_{24}	0.15	–	–	–	–	+	2	1.4
HeLa	1.20	–	–	–	–	+	8	3.3
$CA88LK_{29}$*	0.97	–	+	+	+	+	7	6.1
Medium 1	0	–	–	–	–	–	0	0
Medium 2	0	–	–	–	–	–	0	0
Medium 3	0	–	–	–	–	–	0	0
Medium 4	0	–	–	–	–	–	0	0

*Blind passage.
†CA = caesarean amnion, culture number. CA88 and CA93 were prepared with different batches of human AB serum.

*Difco Laboratories, Detroit, Mich.
†Microbiological Associates, Inc.

J-111: RNA from 6 tubes of J-111, a line derived by Osgood from cells of monocytic leukemia,* and subcultured in our laboratory with 199 solution and 10 per cent calf serum.

EXPERIMENTS AND RESULTS

After removal of the culture fluid, the culture monolayers were briefly washed once with Hanks balanced salt solution, pH 7.1, and then exposed to

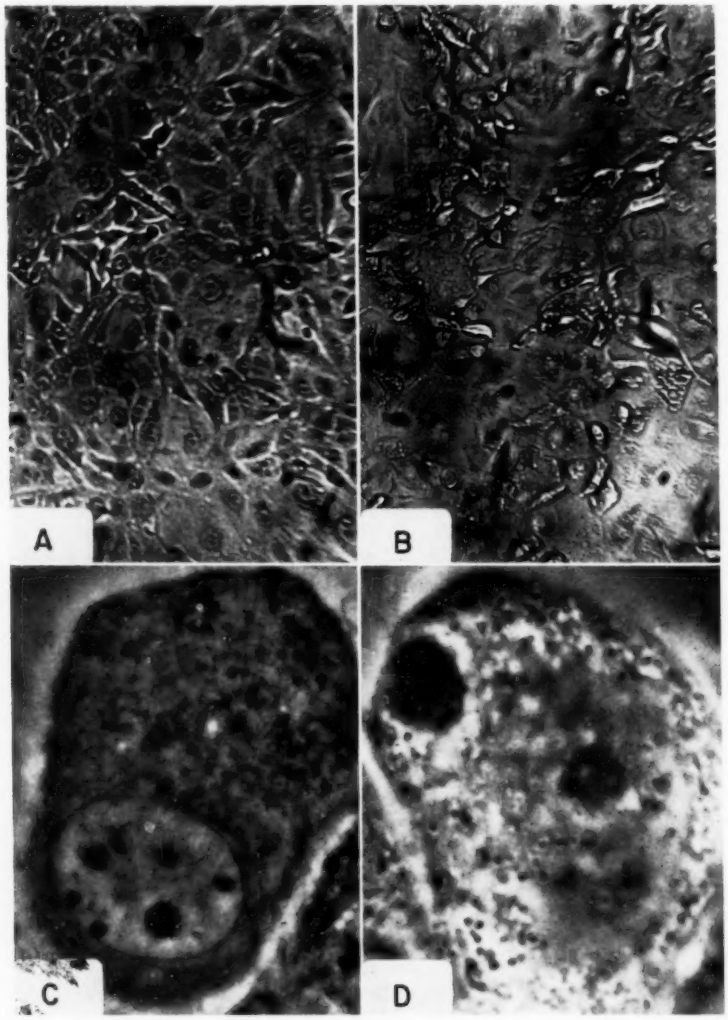

Fig. 2.—*A*, Seven-day-old solid monolayer of primary culture of human amnion growing with 199 solution and 10 per cent of AB human serum. *B*, The same culture 24 hours after addition of leukemic RNA (see text). *C*, A cell from *A*. *D*, A cell from *B*. Cultures grown on cover slips in Leighton tubes; phase contrast of the live cells. In *D* a large cytoplasmic inclusion can be seen at about +300°.

0.2 ml. of inoculum per milliliter of medium, the controls with the same amount (0.2 ml. per milliliter) of medium. After 4 hours at 37° C. stationary, enough medium was added to perform the original medium volume of the tubes or

*Obtained from Microbiological Associates, Inc.

bottles. Readings were made after 24 hours. The changes started with rounding and granularity of the cells which progress to partial, and finally to complete dissociation, of the cell monolayer. The morphologic details of the nucleus and the cytoplasmic inclusions were essentially the same described before,[1-3] that is: the lesions start as in situ cellular lesions without affecting the continuity of the monolayer (CPE++) in about 24 hours. At this stage there is increased refractility of the cellular constituents, sharper demarcation of the cell membrane, and cytoplasmic granularity (Fig. 2, *B*). Cytoplasmic inclusions may be seen (Fig.

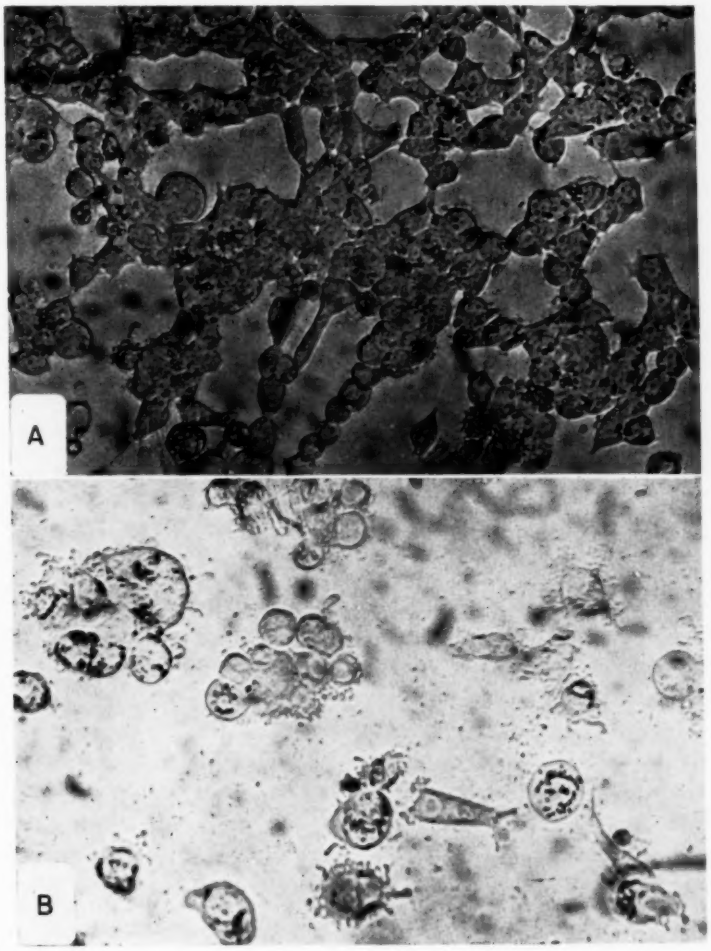

Fig. 3.—Progressive cytopathogenic changes from cultures shown in Fig. 2. *A*, CPE+++. *B*, CPE++++. In *B* there is dissociation of the monolayer, marked cell granularity and discontinuity of the cell membranes with extrusion of cellular materials to the medium.

2, *D*) which are basophilic, but Feulgen-negative. From the third to the tenth day disintegration of the cell monolayer progresses (Fig. 3, *A*, CPE+++) until it becomes complete (Fig. 3, *B*, CPE++++). During these stages the cellular lesions become more severe; carvings of the nuclear membrane, dark lines in the

nucleus, and "explosion" of Feulgen-positive material into the cytoplasm (Fig. 4, *B*) resemble similar changes described by Bernkopf and Rosin[13] in amnion cultures infected with polio, Coxsackie, and ECCHO viruses. The cultures which showed partial dissociation of the sheet (cytopathogenic effect, CPE +++ to ++++) were stored at −20° C. until all of the tubes were ready to be harvested. Fluorocarbon-extracted RNA from these cultures was passed in a similar manner to new cultures. Contrary to previous experiments[2, 3] no EDTA was used throughout.

Table I records the cytopathogenicity of the different inocula with the 4 different media. Positive experiments were considered the ones in which there was complete destruction in more than 50 per cent of the tubes inoculated (CPE_{50}); all the controls remained unchanged.

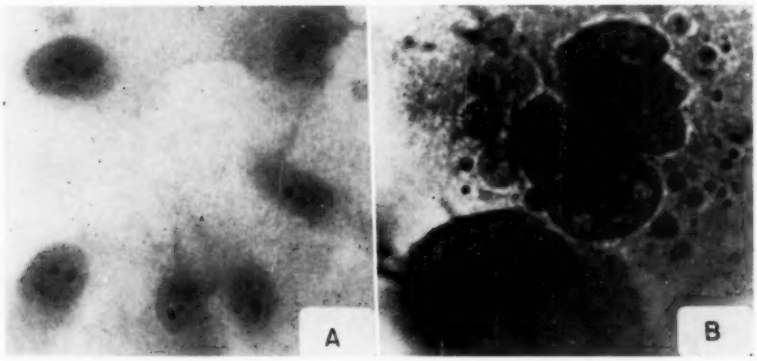

Fig. 4.—*A*, Hematoxylin-eosin staining of a fixed monolayer from a control culture inoculated with RNA from normal leukocytes. *B*, Cell from culture at stage shown in Fig. 3, *B*. Severe nuclear disturbance. Extrusion of nuclear materials (Feulgen-positive) into the cytoplasm.

With the exception of LK_{28}, which seemed to be inactive, all leukemic or malignant inocula produced striking and rapid changes in one or more of the cultural conditions tested. CA88 was a 20-day-old compact monolayer of human amnion culture with vigorous growth and abundant mitoses. Fluid became rapidly acid and had to be changed daily. The culture had been started with heavy seeding (about 10^6 per milliliter). None of the inocula produced changes after 14 days. A culture of similar characteristics grown with a different batch of AB human serum (CA 93) produced some positive results. A blind passage of a negative experiment, $CA88LK_{29}$, was then made with other media and changes were obtained. Again these changes failed to occur when the "inhibitory" batch of serum was used.

Cultures CA90 and CA87 differed from CA88 in that they were much less compact, yet complete sheets, which grew slowly and did not need fluid changes for over 1 or 2 weeks. They were on the alkaline side; the cells were very clear with few mitoses.

Successful passages in media NCTC107 and NCTC109 plus calf serum were obtained with the following treatment of the harvested cultures: (1)

culture fluid, filtered through 03 Selas filter, not homogenized; (2) cells homogenized in the culture fluid at 23,000 r.p.m. for 10 minutes at 4° C., without the fluorocarbon; and (3) cells and culture fluid homogenized several times with trichlorotrifluoroethane[2, 3] at 40,000 r.p.m. for 10 minutes at 2° C.

Aside from showing the effect of different media on the cytopathogenicity of different human leukemic and tumoral inocula, Table I also indicates that:

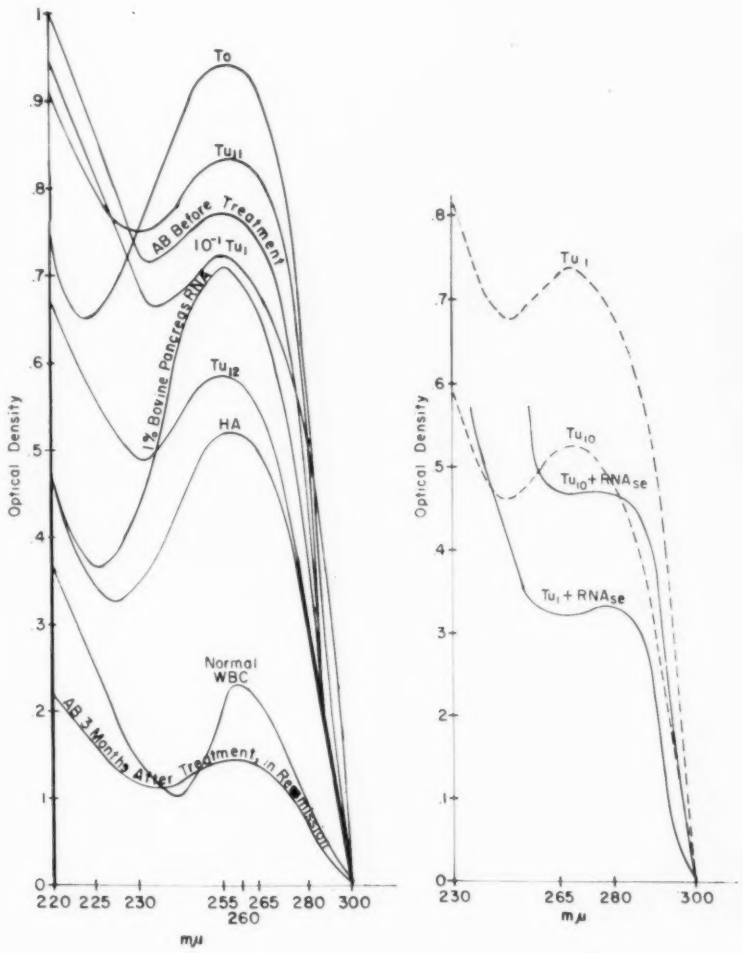

Fig. 4.—*C*, Absorption spectra in the low ultraviolet of fluorocarbon homogenates of 2 acute leukemias in children (*TO* and *AB*), 1 lymphatic leukemia in an adult (*HA*, suspension of 15×10^{10} cells), and 3 solid tumors (TU_1: epithelioid carcinoma of the buccal mucosa; TU_{11}: leiomyosarcoma of the uterus, and TU_{12}: breast carcinoma). *Normal WBC* is a suspension of normal leukocytes containing the same number of cells as in *TO* leukemic preparation. Included is a 1 per cent bovine pancreas commercial preparation of RNA purified by fluorocarbon extraction. The maximum absorption for all preparations is between 257 and 260 while the minimum falls between 228 and 240 mμ. The absorption at 280 mμ is negligible. *D*, Effect of a 1 per cent crystalline, nonproteolytic ribonuclease (NBC) on the absorption spectra of a tumor and a leukemic preparation. After an initial hyperchromic effect of about 30 per cent, the 260 mμ absorption peak decreases markedly. Dialysis was used to remove small molecular chromatic products of digestion. The shoulder at 280 mμ is due to the presence of the enzyme.

(1) RNA from a primary culture ($LK_{29}C$) or a stable culture (J-111) of human leukemic cells behaves in the same manner as the RNA from circulating leukemic cells; (2) RNA from Hodgkin's lymph nodes is cytopathogenic under the conditions of the experiments; (3) RNA from normal cells (leukocytes) or primary cultures of normal origin (amnion, testis*) is not cytopathogenic under the conditions of our experiments; and (4) RNA from stable lines of human malignant origin is cytopathogenic.

Electronmicroscopic study of the RNA preparations in these experiments revealed essentially the same particulate homogenate described in previous works.[2, 3] The homogeniety of the particle size depended on the speed of the homogenization. At 50,000 r.p.m. the particles ranged from 30 to 240 mμ, the intermediate being multiples of 30 (Fig. 5). At 70,000 r.p.m. the range was 30 to 60 mμ.

DISCUSSION

The variables involved in the reproducibility of the cytopathogenic changes are several. Aside from the activity of the inoculum and its instability, which were discussed before,[2, 3] the composition of the medium, the nature of the serum, the rate of growth of the cells relative to the rate of self-duplication of the inoculum, the initial amount of the inoculum, its RNA content, and factors controlling the absorption have to be considered.

From the above experiments, it seems that some batches of human serum were inhibitory even after they were heat-inactivated. The nature of this thermostabile inhibitory activity is not known. Belande and Todd[14] report a thermostabile cytotoxic activity of human adult serum for stable cell lines or cultures of malignant cells. We have tested these inhibitory sera, before and after inactivation, on stable lines and primary lines and also found that they were cytotoxic to stable or malignant lines only, but that the cytotoxicity persisted, or even increased, after heat inactivation. Inhibition by serum was not the only factor (Table I). Slow-growing monolayers in NCTC 107 medium at pH 7.5 with either noninhibitory human serum or calf serum more frequently yielded CPE. The combination of a slow-growing, slightly alkaline culture and pretested calf serum seemed to afford the best results and no failures in passages. Furthermore, for the first time, it allowed passage of unextracted culture fluid.

The blind passage from the noncytopathogenic inoculation would tend to indicate that when the rate of growth of the amnion cells is higher than that of the malignant inoculum, no cytopathogenic effect would be shown, yet the agent is reproducing and released. Beaudreau and co-workers[15] found means of controlling the production of the chicken myeloblastosis virus in culture with and without apparent cytopathogenic effect.

Finally, all other conditions established, there seems to exist a parallelism between the production of CPE and the initial concentration of RNA, the pre-

*An Ep-L culture derived from normal human testis, subcultured 7 times over 6 months

sumably active substance in the inoculum, within a limited period. Concentrations below 0.15 μg per milliliter did not produce any effects under the best conditions.

The cytopathogenicity of viral RNA, the leukemogenic and cancerigenic activity of murine and human leukemic RNA found by other workers, was discussed previously.[2, 3]

Fig. 5.—Electronmicroscopic image of the particulate fluorocarbon-homogenate from 2.5 × 10^7 leukemic stem cells, at 50,000 r.p.m.; gold-palladium shadow casting. The distance between the marks is 1μ. Complete documentation of electronmicroscopic studies, including normal controls and enzymatic characterization of the particles, appears in References 2 and 3.

Dmochovski and associates[16] claim to have obtained transmissible CPE in cultures of monkey kidney with cell-free extracts from human leukemic lymph nodes, and to have obtained increased incidence of murine leukemia with the

same extracts before passage in tissue culture. Leukemogenic and oncogenous activity of human leukemic RNA carried in tissue culture is reported elsewhere.[19] Magrassi[17] reports cytopathogenic changes in a stable human renal cell line and in HeLa with serum filtrates from human patients with leukemia. Given the malignant nature of these lines, the latter results represent a complication rather than a simplification of the experimental scheme and, hence are difficult to interpret.

Bostick's results[18] with fluorocarbon extracts of Hodgkin nodes are closer to ours and his and our electronmicroscopic pictures are very similar, although his extractions are not as extensive. It is unfortunate that in his work, too, only HeLa cultures were used to test the cytopathogenicity of his extracts.

The significance and the nature of the cytopathogenicity of RNA from human neoplastic cells, as opposed to the inactivity of RNA from human normal cells for cultures of human amnion, cannot be established by these experiments alone. If it indicates a viral type of activity, this has to be determined by the study of other viral properties. The fact, however, that none of the normal sources yielded cytopathic effects, makes it unlikely that we were dealing with a property alien to the neoplastic nature of the sources. It requires much further work to establish the nature of the cytopathogenic effect obtained when culture fluid from cultures previously inoculated with fluorocarbon extracted RNA from leukemic tissues is passed without extraction to other cultures. Although this effect was never obtained on first inoculation of the buffer extracts of leukemic tissue, in this case it precludes a free RNA and would tend to point to a more complex particle.

SUMMARY

The reproducibility of the cytopathogenic effect in tissue cultures obtained with human leukemic RNA is increased by the use of slow-growing, though vigorous, alkaline cultures and of noninhibitory human serum or animal serum. These conditions were best met when NCTC 107 and calf serum at pH 7.5 was used instead of 199 and AB human serum at pH 7.2, as previously. The significance of these conditions is discussed.

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BIOLOGIC PROPERTIES OF HUMAN LEUKEMIC AND TUMORAL RNA

IV. Leukemia and Neoplasms Induced in Mice With Human Leukemic RNA Carried in Tissue Culture

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TRANSMISSIBLE tissue culture changes, produced by fluorocarbon extracted RNA from human leukemic and tumor cells, indicated a biologic activity of the "malignant" nucleic acids hitherto unsuspected.[1-4] A natural sequence in the study of the biologic properties of these substances was to determine whether or not human leukemic RNA carried in tissue culture had leukemogenic and/or cancerigenic effects in experimental animals. Lacour and co-workers[5] reported that the intraperitoneal injection of phenol extracted RNA from human leukemic lymph nodes and tonsils into newborn Swiss mice produced, in 3 weeks at the site of injection, mesotheliomas which later metastasized to the peritoneum and lungs. Induction of leukemia and tumors in mice with murine leukemic nucleic acids has been reported by Latarjet and co-workers,[6] Hays and associates,[7] and Ríman and Vesely.[8] Increase in the incidence of a spontaneous murine leukemia by injection of cell-free preparations derived from human and tumor tissues has been obtained by Schoolman and co-workers,[9] Bergol'ts,[10] and Timofeevsky.[11] Similar results were obtained in rats by Ríman and Vesely.[12] In Bergol'ts' experiments the "human leukemic factor" was first passed serially in embryonated chicken eggs. More recently Dmochovski and collaborators[13] claimed the production of leukemia and tumors in mice (10 to 20 per cent) with cell-free extracts from human leukemic lymph nodes carried in tissue culture. In the same report, however, it was stated that the incidence of leukemia in the control subjects did not differ significantly from that in the induced ones.

The results of Parnes and Suntsova[14] seem to be more significant. These authors have utilized saline homogenates of splenic tissue, freshly taken at autopsies of leukemic patients (mostly acute leukemias), and injected subcutaneously in newborn mice from a strain (AMN SSSR) with negligible incidence of spontaneous leukemia. While none of the control group showed leukemia, 27 of 174 mice injected had leukemia in 2 months. Leukemia was also seen in the progeny of the survivors. In a recent report Burton and associates[15] described a method of purification which permitted the recovery from human neoplastic tissues of a "tumor factor," which caused neoplasms and leukemia in 29 of 228 mice injected whereas in the control mice spontaneous leukemia

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occurred in 0.1 per cent and tumors were found in none. On grounds of biologic speculation this "tumor factor" is considered the same, whether from human or murine origin.

Although this represents a very heterogenous group of experiments difficult to interpret, it provides pertinent background for the present work.

MATERIALS AND METHODS

The methods of extracting and quantitating the nucleic acid from human leukemic blood and tumors, and the method for culturing human amnion were the same described in detail in other papers.[2-4]

Swiss (SWRJ) and DBA/2J mice* have been both hybrid and inbred for 3 years. The observed incidence of spontaneous leukemia in this colony is 1 (17 months old) in 300 Swiss and none in 175 DBA/2 mice in 3 years. The incidence of tumors is zero.

Fluorocarbon extracted RNA[2-4] from human leukemic cells of Case LK_{26} (acute stem cell leukemia in an untreated adult) was passed in cultures of human amnion in the manner previously described.[2-4] RNA was extracted from these cultures (LK_{26} TC_4) by the same fluorocarbon method. Both original material and the fourth tissue culture passage were inoculated into the mice in the following fashions: (1) subcutaneously in newborn of less than 12 hours (0.1 ml.), (2) intracerebrally (0.1 ml.) and intraperitoneally (0.5 ml.) in 5- to 7-week-old adults, and (3) intravenously and/or intracardially (0.1 ml.) in pregnant females at term, 12 to 24 hours before delivery. The pregnant females sometimes received

TABLE I

MICE	INOCULA	ROUTES	NO. LEUKEMIC : NO. INJECTED	AVERAGE TIME OF APPEARANCE OF LEUKEMIA (MONTHS)	INCIDENCE OF LEUKEMIA (%)
SWRJ	LK_{26}	i. v., p. f.	7:10	0.3	70.0
		s. c., n. b.	8:15	4	54.4
		i. cer., a.	10:17	3	58.8
	LK_{26} TC_4	s. c., n. b.	8*:14	2	57.0
		i. cer., a.	9:13	3	69.2
	NL	s. c., n. b.	0:7		0.0
		i. cer., a.	0:7		0.0
	NHA	s. c., n. b.	0:5		0.0
	$SWLK_{26}$ TC_4	s. c., n. b.	11*:15	1.5	74.4
		i. p. a.	7:10	3	70.0
	Phosphate buffer	i. cer., a.	1:8	5	12.5
	Noninjected (300)		1:0	17	0.3
DBA/2J	LK_{26} TC_4	i. p. a.	7:10	2	70.0
	$SWLK_{26}$	i. p. a.	7:10	2	70.0
	Noninjected (175)		0:0		0.0

i.v., p.f. = intravenous, pregnant females at term, result refers to offspring.
s.c., n.b. = subcutaneous, newborn.
i. cer., a. = intracerebral, adult.
i.p.a. = intraperitoneal, adult.
*Some of these animals also presented pleomorphic lung tumors, cystic kidneys, and subcutaneous fibromixomas.

*Originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor.

more than 1 injection. This last route was sought to circumvent disturbing cannibalism of the newborn. The concentration of RNA in both original and tissue culture passage was adjusted to 1.90 μg of RNA per milliliter.

The following materials were used as controls: (1) fluorocarbon extracted RNA from human nonleukemic leukocytes, NL (1.95 μg per milliliter), (2) the same passed blindly in

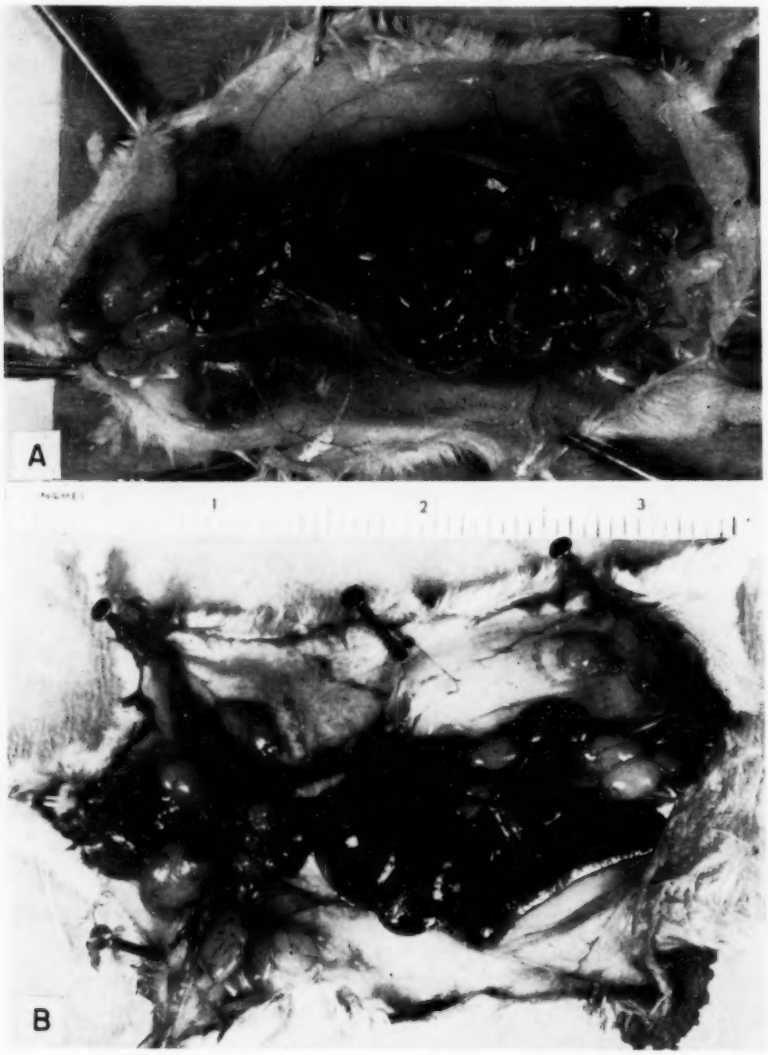

Fig. 1.—Swiss mice injected subcutaneously when newborn. *A*, With the fourth tissue culture passage of the same RNA; lymphadenopathy, hepatosplenomegaly. *B*, With fluorocarbon extract RNA from human leukemic cells; adenopathies, hepatosplenomegaly.

culture of human amnion, NL, TC_n, (3) fluorocarbon extracts of human amnion cultures, NHA (1.90 μg RNA per milliliter), (4) RNA from leukemic lymph nodes of mice injected with tissue culture–carried human leukemic RNA ($SWLK_{28}$ TC_4) [The amounts of RNA in these preparations were not determined.], (5) 15M phosphate buffer, pH 7.2, and (6) uninjected litters from inbred parents.

Table I shows the results of these experiments. The leukemias obtained were accompanied by generalized lymphadenopathy (nodes ¼ inch diameter), and marked hepatosplenomegaly (Fig. 1, *A* and *B*). Histologic examination of these nodes showed replacement

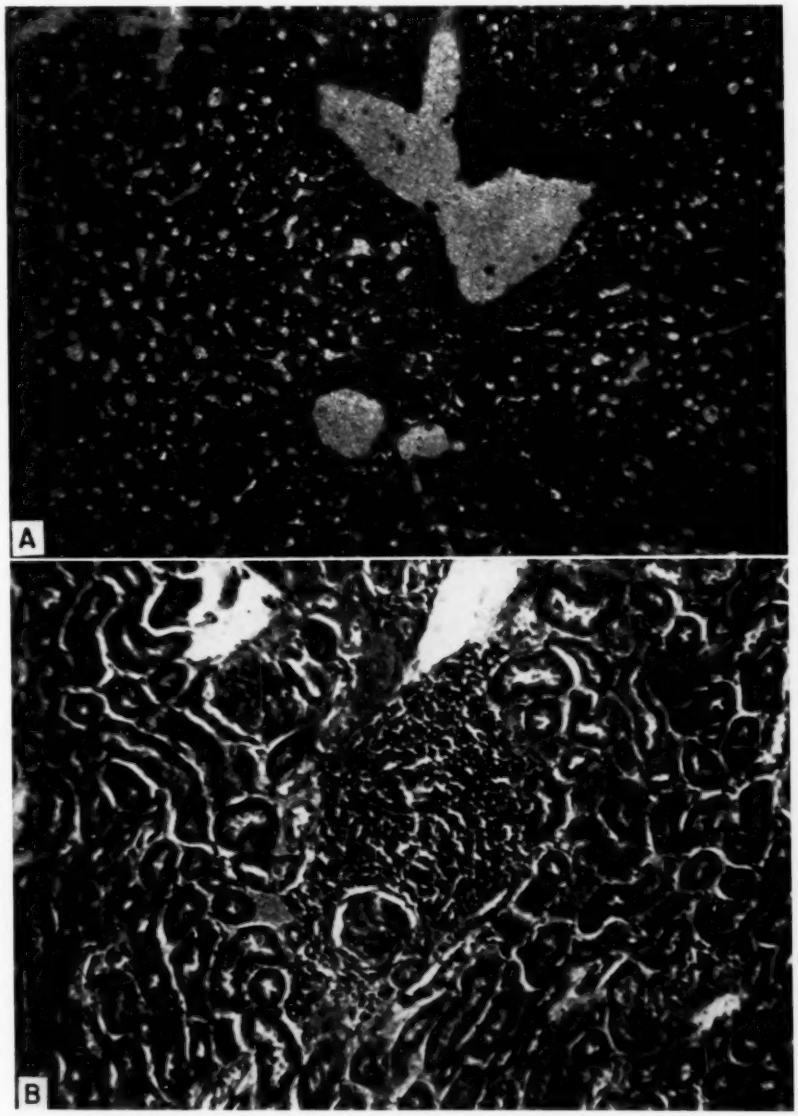

Fig. 2.—Leukemic cells from mice in Fig. 1. *A*, In the peripheral blood. *B*, In imprint of the liver.

of the nodal structure by a monotonous round cell; a homogenous infiltration invaded the capsule. Similar infiltrations were seen in the spleen, liver (Fig. 2, *A*), kidney (Fig. 2, *B*), testis, adrenals, and pancreas. The peripheral blood showed an increased white cell count. These were lymphoblasts with anaplastic features (Fig. 3, *B*). Similar cells were shown in imprints of the liver (Fig. 3, *A*).

Half of the number of the nodes, half of the spleen, and half of the liver were extracted with fluorocarbon[2, 3]; the other halves of these tissues were homogenized in a manual tissue grinder in the cold, with 15M phosphate buffer, pH 7.2. Both of these extracts were injected immediately into mice and were carried in tissue cultures. The harvested fluid from these inoculated cultures was again injected into mice. Leukemia was obtained again after 1 passage in mice and 4 passages in tissue culture: man→tissue culture (4 times)→to Swiss mouse→to tissue culture (4 times)→to Swiss and DBA/2 mice, with both the fluorocarbon and the buffer extracts.

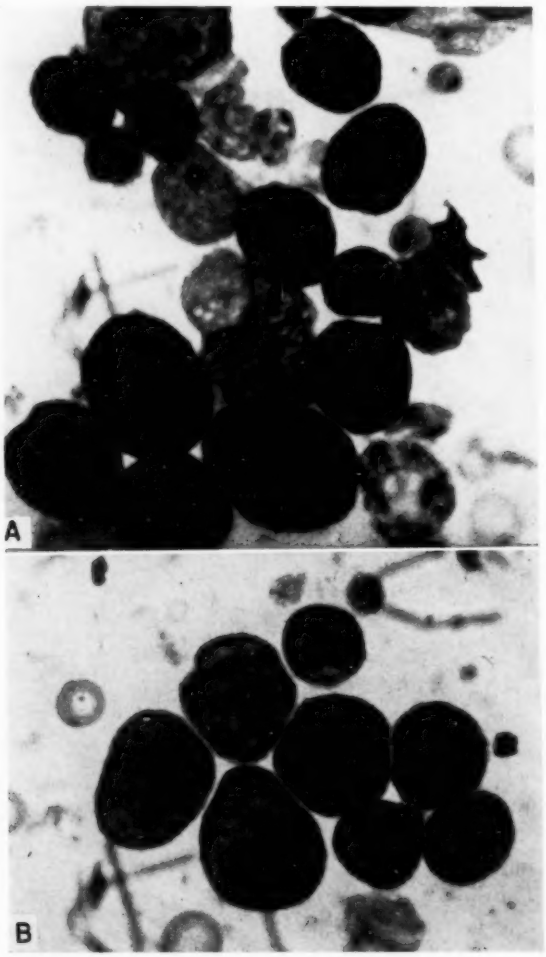

Fig. 3.—Leukemic infiltrations in the organs of the mice in Fig. 1. *A*, Liver. *B*, Kidney.

Two to 3 weeks after injection the animals start showing progressive alopecia on the head and inflammatory changes of the eyes. The fur became rough and rare on the back, and the animals did not gain weight. They could be easily distinguished from normal control animals by these changes and by size and weight, which were notably decreased. They became less and less active and cyanotic, and died from the seventh to the tenth month after the injections.

Besides generalized leukemia, other forms of ill-defined neoplasias have been seen in our injected animals. These included pleomorphic carcinomatous nodules with heavy reticular and plasmyocytic reaction and central necrosis in the lungs (Fig. 4, *A* and *B*), and cystic kidneys. Localized lymphomatous tumors were obtained at the site of injection in DBA/2. Thymic tumors and, in one case, an adenoma of Langerhans islets were noticed.

The leukemic lymphadenopathy was sometimes limited to moderately enlarged regional nodes (paratracheal, intraparotidian, and periaortic) without pronounced hepatosplenomegaly. (Fig. 1, *A*.) In these cases marked reticular hyperplasia of the spleen was noticed, together with microscopic leukemic infiltration of the organs and peripheral blood.

Results of pregnant females injected intravenously at term are not complete yet, and will be reported elsewhere. Death in the offspring as early as at 10 days of age has been observed, with splenomegaly and leukemic infiltrations of the organs.

DISCUSSION

Although leukemic infiltrations of organs, leucocytosis and lymph node enlargement were always present, there was marked variation in the extent of these lesions.

As pointed out by Kassel and Rottino[16] the production of leukemia by cell-free extracts poses the problems of the many variables involved. Reproduction of the fundamental results of Gross and Stewart, for instance, depended on the use of similar sources of materials, extracted by the same technique, administered by the same routes, in the same strains, at the same age. The same authors[16] showed that, all other variables made constant, one can pass from a low incidence of leukemia induction to a high one, by changing to the intravenous route. The smallness of the inocula and the reactive resistance of the host may be responsible for false negative results. Continued subliminal exposure to a cancerigenic virus can induce resistance to massive infection with the same virus.[17]

In what concerns the nature and the source of the methods used by us, our results cannot be compared to any other ones but to themselves only. In order to make this comparison possible, we have used leukemic material from a single patient with leukemia to start with, and maintained a constant concentration of the inocula, only varying the routes and scheme of administration to mice. This would permit an increase in the concentration of the inocula or dilution to the LKD_{50} (50 per cent leukemogenic doses), as the case might be, until constant negative or positive results were obtained.

The only disturbing result in our experiments was the case of leukemia in one animal injected intracerebrally with phosphate buffer. Since it was only one case among the injected, the probability is equal of being a true positive, induced, or a false positive, spontaneous.

The experiments closest to ours are those of Lacour and associates.[5] However, they made use of phenol extracted RNA instead of fluorocarbon. They needed about 500 times more RNA than we did. This was probably due to partial inactivation of the nucleic acid by the method. In our experience phenol extracted RNA from human leukemic cells was not cytopathogenic for tissue cultures in amounts similar to those at which fluorocarbon extracted RNA was.

Ramsey and Stansly[18] reported cancerigenic activity of RNA extracted by fluorocarbon from Hodgkin lymph nodes. This RNA had ultracentrifugal characteristics distinct from RNA from normal tissues.

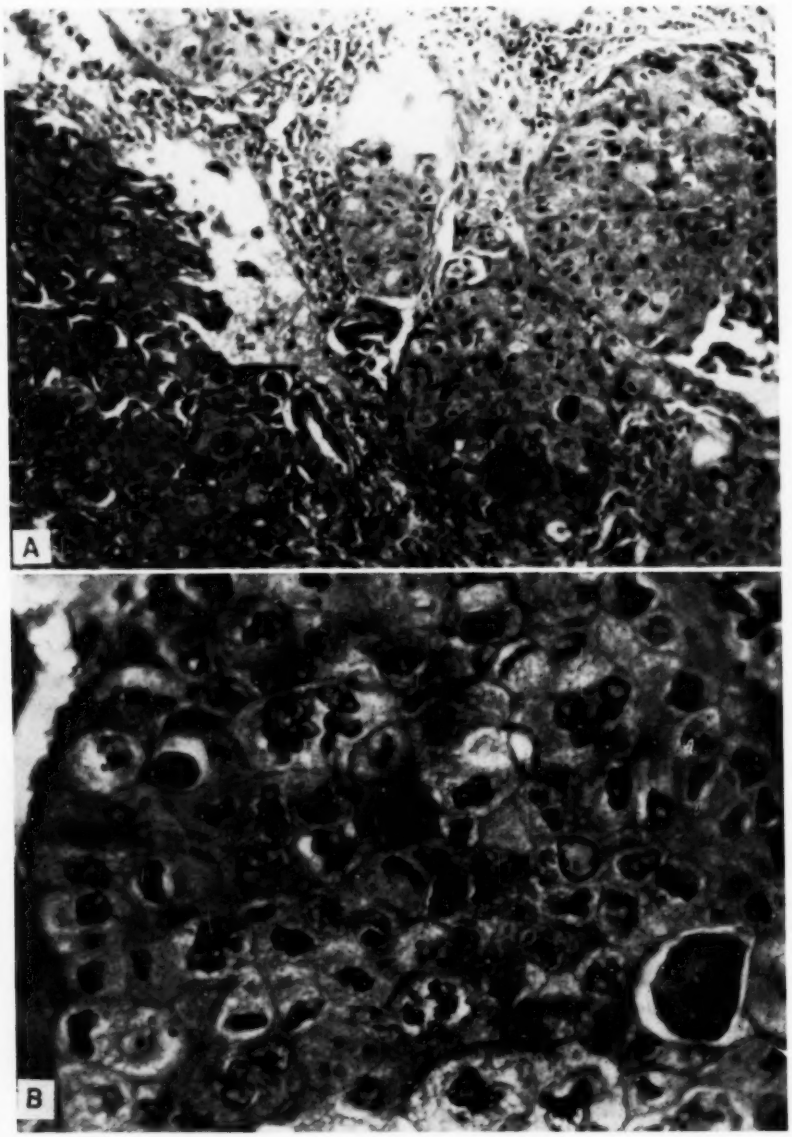

Fig. 4.—Carcinomatous nodules in lungs from mice in Fig. 1. *A*, Central necrosis and surrounding histioplasmocytic reaction. *B*, Magnification from carcinomatous area showing anaplastic features of the nuclei and abnormal mitoses.

The fact that some of the human materials were passed through mice, and recovered again, puts the question of the relationship of the mouse-recovered material, and this only, with polyoma virus. Although the discovery of this

virus shed new light on the problems of cancerigenesis, it is becoming the nightmare of the virus-cancer investigator who has to use rodents. In our case several facts seem to speak against such relationship:

1. Leukemia was obtained directly from man to mice or man to human tissue culture to mouse. Nothing is known of the occurrence of polyoma in human cells.

2. Our material produces mainly leukemia, whether it is passed directly from man to mouse or from man to mouse to mouse, whereas leukemia is practically absent in polyoma infection.[19]

3. We are dealing with a substance that is practically a quasi-protein-free nucleic acid; whereas polyoma is highly resistant to temperature and environmental conditions,[20] our material is easily and quickly inactivated by increasing the temperature above 2° C. or by the presence of traces of heavy metals.

4. When one compares the dynamics and the morphology of the cytopathogenic effects of polyoma virus and our material in a same culture, the differences appear marked. Whereas the cytopathic effect of our materials appears 24 hours after inoculation of the culture and presents inclusions and individual cellular lesions that progress in 5 to 7 days to total destruction, the cytopathic effects of the polyoma virus are late (10 to 18 days) and present a marked agglutination of shrunken, granular cells.

5. Preliminary results, to be presented elsewhere, indicated that an anti-polyoma rabbit serum (provided by Dr. Stewart) does not neutralize the cytopathogenic effect of our preparations and has not prevented the occurrence of leukemia in newborn mice inoculated with material from man to mouse to mouse over 10 months of observation.

SUMMARY

A significant incidence of leukemia and pleomorphic neoplasms in hybrid and inbred Swiss and DBA/2 mice was obtained with tissue culture–carried fluorocarbon extracted RNA from human leukemic cells following various routes and schemes of administration.

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SUSCEPTIBILITY OF RATS WITH DCA HYPERTENSION TO EXPERIMENTAL PYELONEPHRITIS AND AGGRAVATION OF DCA HYPERTENSION BY RENAL INFECTION

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IN PREVIOUS studies we have demonstrated that an experimental model of chronic pyelonephritis can be produced in rats by intracardiac inoculation of bacteria and renal massage, and that these animals fail to develop significant hypertensive vascular disease.[1, 2] Chronic pyelonephritis and hypertension appear related in man; however, data[3-5] which indicate a high incidence of pyelonephritis in individuals with malignant nephrosclerosis are particularly impressive. Rather than an etiologic linkage between pyelonephritis and hypertension, one alternative explanation for the clinical coincidence of these 2 diseases may be that the kidney of the hypertensive individual is more susceptible to renal infection, and that infection may in turn aggravate the hypertensive vascular disease. The present study was undertaken to test this hypothesis. Rats made hypertensive by the administration of desoxycorticosterone acetate (DCA) and a high sodium intake were used as the experimental models.

MATERIALS AND METHODS

Three separate experiments were performed:

I. *Susceptibility of Rats With DCA Hypertension to Escherichia coli Pyelonephritis.*—In earlier studies it was noted that renal massage after inoculation of *E. coli* resulted in a high incidence of pyelonephritis, whereas inoculation of *E. coli* without renal massage rarely produced gross pyelonephritic lesions or infected kidneys.[6] Since the absence of significant pyelonephritis in the kidneys of normotensive rats inoculated without renal massage was so uniform a finding, it was considered that the susceptibility in hypertensive rats could best be studied by a comparison of the results of inoculation of *E. coli* without massage in normotensive and hypertensive rats.

Carworth rats which weighed 80 to 120 grams were started on a diet of rat chow with 1 per cent saline for drinking water. They were then given desoxycorticosterone trimethylacetate* intramuscularly in weekly or semimonthly doses of 5 to 10 mg. until their systolic blood pressure level reached 140 mm. Hg or above. This criterion was achieved in from 12 to

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*Percorten trimethylacetate, supplied by Dr. C. H. Sullivan of the Ciba Pharmaceutical Products, Inc., Summit, N. J.

20 weeks and required a total dose of DCA of from 35 to 50 mg. Twenty-two of the hypertensive animals were then inoculated intracardially with 0.5 ml. of an 18 hour tryptose broth culture of a strain of *E. coli* obtained and maintained as described in previous publications.[2, 6] The kidneys were not massaged. Seventeen normotensive control rats of the same age and weight as the hypertensive animals were simultaneously and similarly inoculated without renal massage.

As an additional control, 14 hypertensive rats and 18 normotensive animals were also inoculated with the same strain of *E. coli;* in these 2 groups the kidneys were massaged through the intact body wall for 5 minutes.

Surviving animals were killed 2 weeks later. The kidneys were examined grossly for pyelonephritic lesions, and one half of each was cultured by means of quantitative techniques which permitted a 1 plus to 4 plus grading of the intensity of infection.[2, 6]

II. *Effect of Pyelonephritis Produced by E. coli on the Severity of DCA Hypertension.*—Thirty Carworth rats which initially weighed 80 to 120 grams were begun on 1 per cent saline and rat chow. Starting 1 week later, they were given a total of 31 mg. of DCA in 5 divided doses. In contrast to Experiment I, in which the dosage was individually titrated for each rat, all animals in this experiment received the same dose at the same times (Fig. 2). Six weeks after the experiment was begun, potassium chloride (35 mEq. per liter) was added to the 1 per cent saline drinking water and was continued throughout the remainder of the study. At the end of 10 weeks, 22 rats who had survived were divided into 2 groups matched for blood pressure and weight. The experimental group then was chosen by coin toss. Each of the 12 rats in this group was inoculated by the intracardiac route with 0.5 ml. of an 18 hour tryptose broth culture of the same strain of *E. coli* used in Experiment I, and each was subjected to renal massage; each in the control group of 10 hypertensive rats was massaged but not inoculated. Periodic blood pressure determinations were continued and all animals which survived were killed 6 weeks later.

III. *Effect of Pyelonephritis Produced by Proteus morganii on Severity of DCA Hypertension.*—Since, in previous studies, Proteus produced a more destructive and persistent renal infection than *E. coli*,[6] Experiment II was repeated employing an 18 hour tryptose broth culture of *P. morganii* diluted 1:2 just prior to inoculation. The strain of Proteus used was obtained as described in previous studies.[2, 6] The experimental design was essentially identical to Experiment II, with these exceptions: (1) a total of 34 rats, which represented the survivors of an initial group of 50, constituted the hypertensive group which was studied; (2) the animals were matched and divided into a group of 18 rats which were inoculated and 16 which were not inoculated, in the same fashion as in Experiment II; this was done 14 weeks after saline and DCA had been started, rather than 10 weeks; (3) all animals received KCl supplement (35 mEq. per liter) from the beginning of the study; and (4) in order to further intensify the pyelonephritis, a second inoculation of the same strain of Proteus, without dilution, was given to the experimental group 4 weeks after the first inoculation. Renal massage in all rats accompanied both inoculations. Survivors were killed 8 weeks later, or 12 weeks after the first inoculation.

General Methods.—Systolic blood pressure in all experiments was measured by the tail plethysmographic method with the rats under light ether anesthesia.[1, 2] In addition, in Experiments II and III the plethysmographic blood pressure determinations were periodically compared with pressures obtained by auscultation of the tail with an Infraton pickup.* With this technique, pulsations appear as pressure is released in a cuff on the rat's tail and are then recorded on a Sanborn electrocardiograph. The latter method revealed identical results to those obtained by the water plethysmograph.

At the time of death blood urea nitrogen (BUN) was measured on whole blood obtained by cardiac puncture. The hearts were trimmed to the ventricles, rinsed in saline, blotted, wrapped in parafilm, and weighed on a balance sensitive to 0.1 mg. The gross lesions

*Medical Electronics Development Company, Great Neck, Long Island, N. Y.

of the kidney in Experiments II and III were rated on a 1 plus to 4 plus scale which has been described previously.[2] One half of each kidney was cultured, and histologic sections stained by the hematoxylin-eosin method were prepared in most instances from the one half of the kidney which remained. Blood cultures were performed in all rats. Additional details of bacteriologic techniques have been described in earlier publications.[6]

RESULTS

Experiment I.—Fifteen rats (21 per cent) died within the first 48 hours after bacterial inoculation, presumably because of the bacteremic shock; these deaths were distributed equally among all 4 groups. The incidence of pyelonephritis in the animals which survived is indicated in Table I. Gross lesions

TABLE I. COMPARISON OF INCIDENCE OF ACUTE PYELONEPHRITIS IN NORMAL RATS AND IN RATS WITH DCA HYPERTENSION (2 WEEKS AFTER E. COLI INOCULATION)

GROUP	KIDNEYS EXAMINED	GROSS PYELONEPHRITIS	%	POSITIVE CULTURES	%	POSITIVE CULTURES > 1+	% > 1+
Hypertensive without massage	32	10	31	13	41	10	31
Normal without massage	30	0	0	6	20	1	3
Hypertensive with massage	24	18	75	16	67	10	38
Normotensive with massage	26	14	54	17	65	9	38
χ^2 [all groups]		35.90	—	16.80	—	11.80	—
p [d (f) = 3]		< 0.01	—	< 0.01	—	0.01	—
χ^2 (H8 vs. N8)		11.10	—	3.11	—	8.23	—
p [d (f) = 1]		< 0.01	—	0.08	—	< 0.01	—

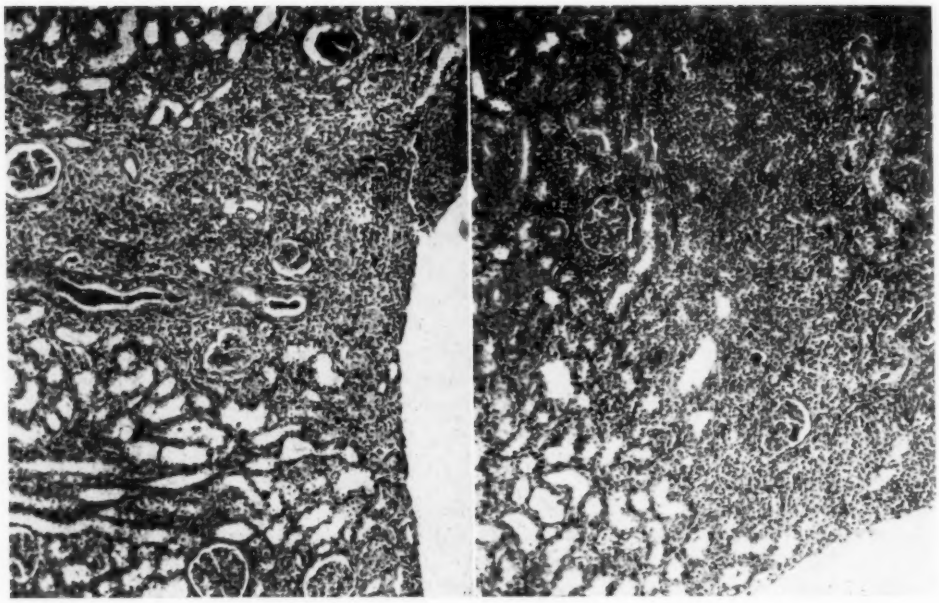
were absent in the kidneys of normotensive, unmassaged animals, a finding which confirmed our previous observations,[6] but were present in 31 per cent of the unmassaged kidneys of hypertensive rats. The lesions typically were wedge-shaped scars which usually extended from the cortex through to the pelvis of the kidney. Histologically, they were identical to those noted in this and previous studies in massaged normotensive rats (Fig. 1). The incidence of gross lesions in massaged kidneys of normotensive rats in this study was 54 per cent; this was increased to 75 per cent in the massaged kidneys of hypertensive rats.

TABLE II. BLOOD PRESSURE AND BODY WEIGHT PRIOR TO INOCULATION, AND HEART WEIGHT AT AUTOPSY IN RATS IN EXPERIMENT I*

GROUP	SYSTOLIC BLOOD PRESSURE (MM. HG)	BODY WEIGHT (GM.)	HEART WEIGHT (MG./100 GM.)
Hypertensive without massage	149 ± 9	337 ± 33	348 ± 38
Normotensive without massage	111 ± 12	352 ± 40	252 ± 18
Hypertensive with massage	145 ± 13	329 ± 41	346 ± 37
Normotensive with massage	113 ± 15	349 ± 42	253 ± 14

*The weights and blood pressures presented in this table and in Tables III and IV indicate the mean and standard deviation of each group.

In 8 kidneys from unmassaged hypertensive rats which did not show pyelonephritic lesions grossly, there were microscopic manifestations of inflammation. This made a total of 18 of 32 kidneys (56 per cent) with evidence of pyelonephritis. The occasional positive cultures in the unmassaged normotensive



A. *B.*

Fig. 1.—*A*, Section through cortex and part of medulla of hypertensive rat inoculated with *E. coli* without massage, 2 weeks prior to death. (Magnification ×140; reduced ½.) *B*, Similar area of pyelonephritis in normotensive rat inoculated with *E. coli* and subjected to renal massage. (Magnification ×140; reduced ½.)

rats usually consisted of only a few organisms. They were associated in a rare instance with small interstitial focal accumulations of mononuclear cells, but never with the marked and diffuse polymorphonuclear and mononuclear infiltration noted histologically in the unmassaged hypertensive rats or in massaged kidneys.

In the animals which received DCA, minimal dilatation of the collecting tubules in the outer zone of the medulla and slight swelling of the epithelial cells were noted, suggesting the changes of potassium deficiency which have been pointed out by Oliver and his co-workers.[7]*

In Table II, the averages of the body weight and blood pressure at the time of inoculation, and the heart weight at death 2 weeks later, have been tabulated. The differences between the groups in blood pressure prior to inoculation and the relative heart weights at autopsy were those which would be expected. Body weights at the time of inoculation were not significantly different. Blood cultures were sterile in all rats.

Experiment II.—The 12 experimental rats had a mean systolic blood pressure of 143 ± 19.9 mm. Hg and a mean body weight of 333 ± 46 grams immediately prior to inoculation and renal massage; the 10 rats who were not inoculated had a mean blood pressure of 146 ± 14.3 mm. Hg and a mean weight of

*We are indebted to Dr. M. A. Holliday, Department of Pediatrics, University of Pittsburgh School of Medicine, for assistance in interpreting the renal changes caused by DCA.

331 ± 56 grams. Four experimental animals with an average preinoculation pressure of 148 mm. Hg, and 1 control with a preinoculation pressure of 145 mm. Hg, died during the first week. The subsequent course of the blood pressures of the 8 infected and 9 uninfected survivors is indicated in Fig. 2. The infected animals showed a significantly higher pressure than the uninfected animals 10 days after the inoculation ($p = 0.001$); thereafter, blood pressure became identical in both groups.

In accord with the return of blood pressure to previous levels in the infected group were the findings from the kidney cultures obtained at autopsy 6 weeks after inoculation (Table III) which indicated that the infection was subsiding. Over one half of the kidneys had become sterile, whereas those which were still infected showed relatively few organisms (2 plus, or less, with an intensity of infection index of 0.11). The disappearance of organisms from the pyelonephritic kidneys is in keeping with earlier studies in which it was demonstrated that *E. coli* infection is largely self-limited, and subsides in 6 to 12 weeks.[6] All blood cultures were sterile.

Gross pyelonephritic lesions in various stages of healing were present in 75 per cent of the kidneys of inoculated animals; these were of only moderate severity with a severity index of 0.42. Heart weight and BUN were not significantly different between the 2 groups, a fact which lends further confirmation to the observation that the elevation of blood pressure in the infected group was a transient phenomenon, and existed only at the height of the infection.

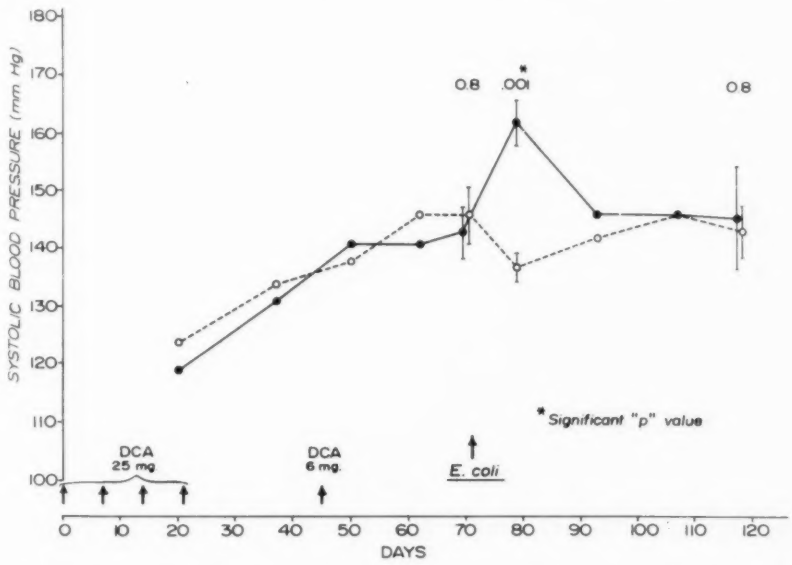


Fig. 2.—Effect produced on blood pressure of rats with DCA hypertension by infection with *E. coli* (●——●), contrasted with the uninoculated control hypertensive group (○ - - - ○). Although the groups were matched for equal blood pressures just prior to inoculation (see text), means of the preinoculation blood pressures for the 12 experimental animals and 10 control hypertensive animals have been calculated, and the curves have been extrapolated back to the start of the experiment. After inoculation of *E. coli*, each pair of points represents the mean of 8 infected or 9 uninfected rats. Standard errors are indicated where appropriate.

TABLE III. RESULTS AT AUTOPSY 6 WEEKS AFTER INFECTION OF HYPERTENSIVE RATS WITH E. COLI

GROUP	NO. OF RATS	RENAL CHANGES						FINAL BODY WEIGHT (GM.)	HEART WEIGHT (MG./100 GM.)	FINAL SYSTOLIC BLOOD PRESSURE (MM. HG)
		TOTAL KIDNEYS EXAMINED	INFECTED KIDNEYS	INTENSITY OF INFECTION INDEX*	KIDNEYS WITH GROSS PYELONEPHRITIS	SEVERITY OF LESIONS INDEX†	BUN (MG./100 ML.)			
Inoculated	8	16	7	0.11	12	0.42	21.1 ± 7.6	365 ± 35	320 ± 26	146 ± 25
Not inoculated	9	18	0	—	0	—	22.8 ± 2.7	365 ± 44	322 ± 32	143 ± 14
"p"							> 0.05	> 0.05	> 0.05	> 0.05

*Intensity index = $\frac{\text{sum of grades of infection}}{\text{number of kidneys} \times 4}$; infection is graded on a 1 plus to 4 plus scale, depending on amount of growth on culture plate.[2]

†Severity index = $\frac{\text{sum of grades of gross lesions}}{\text{number of kidneys} \times 4}$; gross lesions are graded on a 1 plus to 4 plus scale, depending on the extent of gross pathologic damage.[2]

TABLE IV. RESULTS AT AUTOPSY 12 WEEKS AFTER INFECTION OF HYPERTENSIVE RATS WITH PROTEUS MORGANII

GROUP	NO. OF RATS	RENAL CHANGES							FINAL BODY WEIGHT (GM.)	HEART WEIGHT (MG./100 GM.)	FINAL SYSTOLIC BLOOD PRESSURE (MM. HG)
		TOTAL KIDNEYS EXAMINED	INFECTED KIDNEYS	INTENSITY OF INFECTION INDEX*	KIDNEYS WITH GROSS PYELONEPHRITIS	SEVERITY OF LESIONS INDEX*	RATS WITH RENAL STONE	BUN (MG./100 ML.)			
Inoculated	14	28	28	0.91	27	0.70	6	23.2 ± 3.6†	359 ± 57	337 ± 39	165 ± 19†
Not inoculated	16	32	0	--	0	--	0	20.3 ± 2.5	384 ± 43	297 ± 17	138 ± 19
"p"								0.02	0.2	0.01	0.001

*See notes to Table III.
†Thirteen rats only.

Examination of the histologic sections revealed typical subacute pyelonephritic lesions in the inoculated group. Three of the 4 kidneys which did not show gross lesions did demonstrate microscopic evidence of inflammation. Vascular lesions which resulted from DCA consisted of medial hypertrophy of small arteries with hyperplastic changes in a few arterioles, and occasional areas of hyaline degeneration in glomeruli. These vascular changes were noted in both groups with no obvious increase in severity in the infected animals.

Experiment III.—The mean blood pressure and body weight in the experimental group of 18 rats at the time of inoculation and massage were 136.1 ± 15.3 mm. Hg and 326 ± 38 grams, respectively; in the group of 16 rats massaged but not inoculated, these values were 136.9 ± 20.2 mm. Hg and 315 ± 36 grams. The slightly lower preinoculation blood pressures in this experiment, as compared to those in Experiment II, may have been a result of the administration of KCl throughout the course of the study.[5]

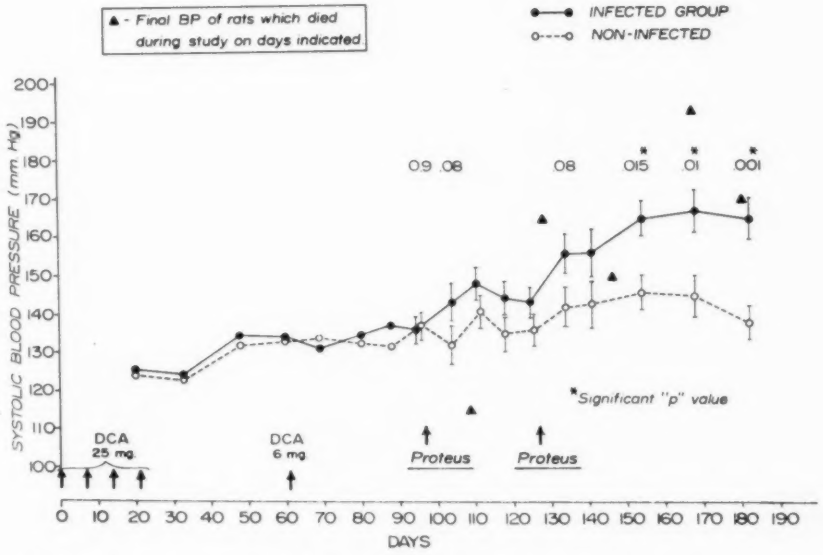


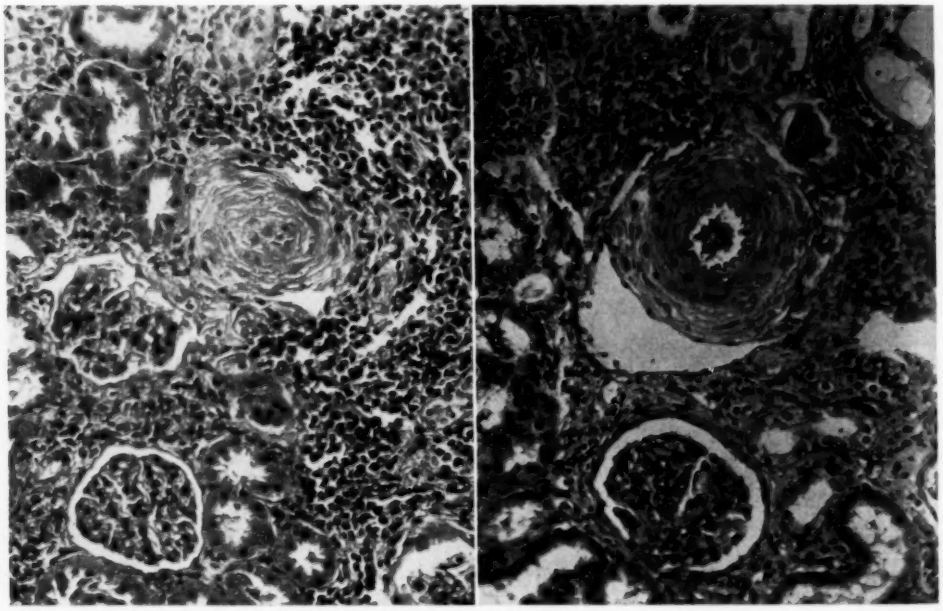
Fig. 3.—Effect produced on blood pressure of rats with DCA hypertension by infection with *P. morganii* (●——●), contrasted with the uninoculated control hypertensive group (o- - -o).

As in Fig. 2, the curves for each group have been extrapolated back to the start of the experiment, and each pair of points represents either 18 experimental animals or 16 control hypertensive animals. After the inoculation of Proteus, the blood pressures of the infected rats which died (▲) are included in the mean just prior to their death, and "n" is accordingly reduced by 1 in the succeeding mean. Final "n" for the infected group is 13, 1 rat having been killed 2 days prior to the termination of the experiment (see text). The number of rats in the noninfected control hypertensive group remains at 16 throughout the experiment. Standard errors are indicated where appropriate.

The blood pressure curves are indicated in Fig. 3, and the results at autopsy are set forth in Table IV. Four rats in the inoculated group died during the 12 weeks, and 1 was killed 2 days prior to the termination of the experiment; the last pressure before death in these 5 is also indicated in Fig. 3. None of the control hypertensive rats died. A slight, but statistically insignificant, rise

in blood pressure developed in the infected group and persisted for at least 1 month. After the animals received a second inoculation, this difference became considerably more marked and reached a high level of significance ($p = 0.001$) at the end of 12 weeks.

In contrast to the autopsy findings in Experiment II, all the kidneys from the inoculated rats remained heavily infected with Proteus with an intensity of infection index of 0.91. Severe lesions of gross pyelonephritis were present in all but 1 of the kidneys from the inoculated rats and many contained stones.[2] As indicated by the severity index of 0.70, this renal damage was somewhat worse than that demonstrated by the rats infected by *E. coli* in Experiment II.



A. *B.*

Fig. 4.—*A*, Medium sized arteriole in infected rat from Experiment III indicating marked hyperplastic sclerosis and occlusion of the lumen. Final blood pressure in this rat was 200 mm. Hg. (Magnification ×280; reduced ⅓.)
B, Artery, probably interlobular, from same animal demonstrating marked medial hypertrophy. (Magnification ×280; reduced ⅓.)

Only 1 rat had a positive blood culture; it was in a terminal state and was killed 2 days earlier than the remainder of the group. Blood pressure in this animal had been in the range of 150 to 170 mm. Hg, but was unobtainable on the day of autopsy; BUN was 94 mg. per cent. Despite the omission of this rat from the mean, the BUN was slightly, but significantly, higher in the infected group ($p = 0.02$). Heart weight relative to body weight was significantly elevated in the infected group ($p = 0.01$) whereas final body weights were not significantly different.

Histologic sections of the kidneys revealed vascular lesions which resulted from DCA administration as described in Experiment II. However, although

not evaluated statistically, hyperplastic sclerosis of arterioles, manifested by intimal thickening, medial hypertrophy, and marked narrowing of the lumen, appeared considerably more prominent and severe in the kidneys of the infected group. In several infected rats these arteriolar lesions were quite similar to those which Saphir and Taylor[5] have noted in malignant hypertensive subjects and have considered as characteristic of pyelonephritis lenta (Fig. 4).

DISCUSSION

Chronic pyelonephritis has been widely held to be one of the primary causes of hypertensive vascular disease. Although many other diseases and conditions are associated with an increased incidence of pyelonephritis,[9] only in hypertensive disease has this association been considered an etiologic one. The emphasis on pyelonephritis as a possible cause of hypertensive disease, rather than the consideration that the latter disease may be a factor involved in the pathogenesis of pyelonephritis, as, for instance, in diabetes mellitus and pregnancy, is probably the result of the important role which the kidney has been assigned generally in the etiology of hypertension. However, there are several inconsistencies in the assumption that pyelonephritis plays a direct role in the development of hypertensive disease. Hypertension is not a feature of acute pyelonephritis, as is the case in acute glomerulonephritis. A clear correlation between degree of renal impairment in pyelonephritis and severity of hypertensive disease has not been demonstrated; indeed, although Weiss and Parker,[4] and Saphir and Taylor[5] suggest that chronic pyelonephritis may result in malignant hypertension, others point out that the hypertension of pyelonephritis is mild.[10] Clinically it is not uncommon to see patients who succumb to renal failure, caused by proved pyelonephritis, manifesting normal blood pressure and no evidence of heart disease.[3] We have been impressed, moreover, by the similarity in the clinical picture presented by patients with primary hypertension and those in whom the etiology has been attributed to chronic pyelonephritis because of a history of urinary tract infection.

These conflicting data first led us to the study of chronic pyelonephritis in the rat employing an experimental model in which the effects on the blood pressure of chronic infection alone could be assayed. Studies previously reported demonstrate decisively that in the rat the parenchymal renal damage which results from chronic pyelonephritis alone does not produce vascular disease or hypertension.[1, 2, 12] Insofar as animal studies can be applied to the human disease, the present experiments may thus provide an alternative explanation to the etiologic one, for the clinical relationship between pyelonephritis and hypertension, an explanation perhaps more in keeping with existing clinical information. Thus, (1) the kidney of the hypertensive subject may be more prone to infection than that of the normal subject; and (2) once established, infection may aggravate the hypertensive disease.

DCA hypertension cannot be compared easily to human hypertensive disease and represents but one of several types of experimental hypertension. Moreover, other variables which may have influenced susceptibility are present in Experiment I. For instance, potassium deficiency which may have resulted

from the administration of DCA has been suggested as a cause of increased susceptibility to renal infection.[13] Attempts to demonstrate such increased susceptibility in normotensive rats on potassium deficient diets, however, have revealed results which are diametrically opposed in 2 different studies.[14, 15] Moreover, simultaneously with our studies, Woods[16] has demonstrated identical results to ours in rats with DCA hypertension, in an experiment in which potassium balance was rigorously and successfully maintained. It may be pertinent that, among patients with potassium deficiency, Relman and Schwartz[17] have pointed out that the incidence of pyelonephritis appears to be highest when the potassium depletion is secondary to primary aldosteronism, a condition usually associated with hypertension.

Secondly, trapping of bacteria in the kidney in animals given DCA may be related to the fact that these kidneys are swollen and enlarged. This may be equivalent to the temporary engorgement and diminished clearance of bacteria produced by our technique of renal massage.[18] The development of intrarenal hydronephrosis, which Freedman and Beeson[19] have shown to be pathogenetically important in pyelonephritis, could also be a factor in the increased susceptibility described in Experiment I. Additional studies, which should include determination of susceptibility to pyelonephritis in animals with forms of hypertensive disease other than that caused by DCA, are necessary to clarify these issues.

The demonstration of an increase in the severity of hypertension by renal infection in our studies seems clear-cut. With *E. coli*, which produced self-limited infection, the increase in blood pressure was transient and unassociated with permanent change. With Proteus, which resulted in persistent infection and more severe renal damage, the increased blood pressure was permanent and associated with increased heart size, a slight rise in BUN, and vascular damage which suggested that seen in malignant hypertension. These findings are in accord with those of the biopsy studies of Merriam, Sommers, and Smithwick,[11] which indicate that pyelonephritis is associated with a more severe grade of hypertension, lend confirmation to the hypothesis espoused by Goldblatt[20] that pyelonephritis may be a cause of acceleration of hypertensive disease, and are quite compatible with the autopsy findings of Weiss and Parker, and of Saphir and Taylor that a high incidence of pyelonephritis is present in malignant hypertension. Clinically, the implications of these studies offer a further important rationale for the industrious search and intensive treatment of active pyelonephritis in any stage of hypertension, and particularly in those patients with accelerated forms of the latter disease.

SUMMARY

Hypertension was produced in rats by the administration of desoxycorticosterone and saline. Inoculation with *Escherichia coli*, with no additional renal manipulation, produced evidence of pyelonephritis in the kidneys of approximately one half of these hypertensive rats, whereas the incidence in normotensive rats was negligible. In separate experiments, temporary aggravation of

DCA hypertension by production of a transient renal infection produced by *E. coli*, and permanent progression of hypertensive disease by severe renal infection produced with *Proteus morganii* were demonstrated.

Mrs. Helen Busony and Miss Mary Kaufman gave additional technical assistance in various phases of the work.

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EVALUATION OF THE HEMAGGLUTINATION REACTION AS A DIAGNOSTIC TOOL IN INFECTIOUS HEPATITIS

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After significance of Porterfield's hemagglutination reaction in yellow fever was demonstrated[1] attempts were made to ascertain whether or not a similar reaction occurred in other virus diseases of man associated with jaundice, namely, infectious and serum hepatitis. Hoyt and Morrison,[2] in 1956, reported that serum of patients with infectious hepatitis contained agglutinins reactive with suitably prepared monkey *(Macaca rhesus)* erythrocytes which were far in excess of those encountered in control specimens. In 19 cases of infectious hepatitis where single specimens were obtained, presumably in the acute phase of illness, titers of 1:8 to 1:32 were observed, whereas in a control group of 125 blood donors, all but 15 were observed to have titers of less than 1:8. In 1957, Rubin and co-workers[3] in a series of 51 patients with clinically active hepatitis observed that all showed agglutinins reactive with rhesus monkey erythrocytes in titers exceeding 1:128, whereas, in an unspecified number of control subjects, titers ranged from less than 1:4 to 1:64. Havens,[4] in 1958, studied a series of 54 patients with infectious hepatitis from whom single serum specimens were obtained during the first 17 days of illness and observed in 47 of these patients hemagglutinins reactive with 1-day-old chick erythrocytes in titers ranging from 1:40 to 1:1,280. In similar tests with sera obtained from 56 normal subjects, only 9 individuals possessed titers as high as 1:40 and 1:80.

In view of the encouraging results obtained in tests to measure serum levels of chick and monkey erythrocyte agglutinins in serodiagnosis of infectious hepatitis, attempts were made in our laboratory from 1956 to 1958 to prepare the so-called hepatitis hemagglutinin and to evaluate it in an extensive series of tests for the laboratory diagnosis of human hepatitis in United Nations troops stationed in the Far East. In this article we will present a summary of our data.

MATERIALS AND METHODS

Sera.—Sera used in these studies were obtained from 200 patients with clinically diagnosed hepatitis in United Nations military hospitals in Japan, Korea, and Okinawa. In addition, serum from 64 patients with a variety of serologically proved or clinically diagnosed diseases and from 100 blood donors who remained well after being bled were used for control purposes. Sera were used immediately after collection or after storage at -20° C. in plastic-capped glass tubes for 1 day to 6 months.

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Preparation of Hemagglutinin.—In general, extraction of serum to be used as sources of hemagglutinin or as control materials was performed according to Casals' method[5] as modified by Porterfield.[1] The extraction procedure was employed to remove inhibitors which in some sera masked presence of chicken erythrocyte agglutinins. Serum in 2 ml. amounts was thoroughly mixed by syringe and needle with 30 ml. of chilled acetone. The mixture was centrifuged at low speed and the sedimented precipitate was washed 6 times in acetone, twice in equal parts acetone and anhydrous ether, and once in ether. Washed precipitate was dried *in vacuo* and the resultant powder was resuspended in 6.0 ml. of phosphate buffered saline (pH 7.5). The extract was examined for hemagglutinating activity at once, or after 1 or 2 days storage at −20° C.

Preparation of Chick and Monkey Red Blood Cells and Technique of Hemagglutination Test.—Blood was harvested in Alsever's solution from 1- to 5-day-old chicks, and the cells were washed 3 times in physiologic saline solution. The volume of cells was measured after centrifugation in a graduated tube in a horizontal machine at 2,000 r.p.m. for 10 minutes, and sufficient phosphate buffered saline, pH 7.5, was added to the cells to make a 0.25 per cent suspension. Cells were used on the day of harvest. Monkey blood was collected in Alsever's solution and was stored at 4° C. for periods up to 1 week. Cells for 1 day's work were washed and prepared in the same manner as were chick cells.

The technique developed during the course of this work and subsequently adopted has been reported in detail[6]; here it is described only briefly. Serum preparations were examined for presence of chick cell hemagglutinin (CCHA) or monkey cell hemagglutinin (MCHA) by mixing 0.25 ml. amounts of serial twofold dilutions with 0.5 ml. volumes of 0.25 per cent chicken or monkey erythrocytes and 0.25 ml. amounts of buffered saline. Satisfactory agglutination was obtained when all reagents employed in the test were buffered at pH 7.5. Mixtures were incubated in the ice box at 4° C. for 90 minutes. Positive tests were considered those in which classic patterns of agglutinated cells (similar to those seen in reactions with influenza virus and chicken erythrocytes) were obtained and negative tests were those in which clumps of agglutinated cells or no agglutinated cells could be seen. For comparative purposes simultaneous tests to detect and measure hemagglutinins were performed with extracted and nonextracted serum against both chicken and monkey erythrocytes.

Hemagglutination Inhibition Test.—Twenty-five hundredths milliliter amounts of twofold dilutions of acute phase serum extracts to be examined for CCHA or MCHA activity were prepared in Kahn tubes with buffered saline used as a diluent. To the diluted material was added 0.5 ml. volume of 0.25 per cent suspension of chicken or monkey erythrocytes and 0.25 ml. amounts of buffered saline after which the mixtures were incubated at 4° C. for 90 minutes and the tests were read. The end point was the highest dilution of serum extract which gave complete agglutination of erythrocytes. Each 0.25 ml. amount of the end point dilution of extract was considered to contain one hemagglutinating unit. Serum to be tested for hemagglutination inhibition activity was inactivated at 56° C. for 30 minutes and serial twofold dilutions were then made with buffered saline used as diluent and were distributed in 0.25 ml. amounts into Kahn tubes. Two units of CCHA or MCHA contained in 0.25 ml. volumes were added to each tube. The mixture was incubated at 37° C. for 30 minutes and then 0.5 ml. of 0.25 per cent chick cells were added. After incubation at 4° C. for 90 minutes, tubes were examined for inhibition of hemagglutination.

RESULTS

Hemagglutination Test Results With Sera From Patients With Infectious Hepatitis, With Infectious Diseases Other Than Hepatitis, and From Normal Individuals.—Data presented in Table I represent results obtained in hemagglutination tests with sera from 20 patients with infectious hepatitis and are illustrative of findings in tests performed over a 27 month period with sera from 200 patients. The diagnosis of infectious hepatitis was established on

TABLE I. HEMAGGLUTINATION FINDINGS IN TESTS WITH CHICKEN AND MONKEY ERYTHROCYTES AND EXTRACTED AND UNEXTRACTED SERUMS IN PATIENTS WITH INFECTIOUS HEPATITIS

GROUP	PATIENT	DD*	SERUM TITERS OBTAINED WITH			
			CHICKEN ERYTHROCYTES		MONKEY ERYTHROCYTES	
			EXTRACTED SERUM	NONEXTRACTED SERUM	EXTRACTED SERUM	NONEXTRACTED SERUM
I	1	18	12	96	24	24
		32	12	24	24	24
		39	0†	12	12	24
	2	16	24	12	192	768
		30	24	12	48	192
		57	0	0	12	192
	3	7	48	96	192	768
		24	24	24	192	384
		47	0	12	48	384
	4	22	6	0	6	0
		36	0	0	6	0
		51	0	0	0	0
	5	23	48	12	384	48
		30	12	12	384	24
		51	12	6	768	12
	6	27	6	0	24	24
		41	0	0	12	24
		55	0	0	24	0
	7	24	12	0	24	12
		31	12	0	24	24
		46	6	6	24	192
	8	1	96	96	ND‡	ND
		38	96	96	ND	ND
		51	0	48	ND	ND
		58	0	24	ND	ND
II	9	13	12	12	24	12
		20	12	24	24	12
		40	12	24	96	96
	10	27	12	0	12	96
		34	ND	0	24	0
		42	12	0	24	0
III	11	19	0	0	192	768
		27	0	0	384	768
		41	0	0	96	1,536
	12	20	0	0	0	24
		41	0	0	0	0
		56	0	0	0	24
	13	13	0	24	0	6
		20	0	0	0	0
		42	0	0	0	0
	14	21	0	0	24	24
		43	0	0	12	24
		57	0	0	12	24
	15	14	0	0	24	24
		23	0	0	0	24
		30	0	0	0	24
	16	1	0	12	24	48
		11	0	0	6	24
		18	0	0	6	12
	17	16	0	0	48	48
		23	0	0	48	48
		36	0	0	24	24

TABLE I—CONT'D

GROUP	PATIENT	DD*	SERUM TITERS OBTAINED WITH			
			CHICKEN ERYTHROCYTES		MONKEY ERYTHROCYTES	
			EXTRACTED SERUM	NONEXTRACTED SERUM	EXTRACTED SERUM	NONEXTRACTED SERUM
	18	17	0	0	192	384
		31	0	0	192	768
		45	0	0	96	384
	19	25	0	0	1,536	3,072
		31	0	0	1,536	3,072
		38	0	0	1,536	3,072
	20	22	0	12	0	96
		29	0	0	12	24
		36	0	0	0	0

Numbers are reciprocals of serum titers.
*DD = day after onset of disease.
†0 = no pattern of agglutinated cells at 1:6 dilution, the lowest serum dilution tested
‡ND = not done.

clinical grounds and on detection of physiologic alteration of the liver by application of standard liver function tests routinely employed in U. S. Army hospital laboratories.[7] These representative data indicate that in tests with extracted sera and chick erythrocytes the most frequent agglutination pattern in patients with hepatitis is high CCHA serum titers in acute illness and low or undetectable CCHA serum levels in convalescence (Table I, Group I, 8 patients). This is followed in frequency by high CCHA titers that persist at elevated levels throughout the course of illness (Table I, Group II, 2 patients). In the remaining patients no CCHA is detectable at any time in the course of illness (Table I, Group III, 10 patients). Normal individuals are found to have either no detectable CCHA (Table II, Group I, 17 individuals) or serum levels of this factor remain at a constant level (Table II, Group II, 3 individuals). In tests with paired sera obtained from patients suffering with mumps (12 patients), leptospirosis (8), poliomyelitis (12), lymphocytic choriomeningitis (8), cirrhosis with jaundice (12), and hemorrhagic fever (12) results were similar to those obtained with normal human sera; in these 64 patients either CCHA was not demonstrable (54 individuals) or was present in both early and late serum specimens in unchanging amounts in titers ranging from 1:6 to 1:24 (10 individuals). Results obtained in tests with sera of patients with infectious mononucleosis (6 patients) were similar to, and not distinguishable from, those observed in tests with sera from patients with hepatitis, i.e., in acute illness in patients suffering with infectious mononucleosis, CCHA was present in titers ranging from 1:6 to 1:24 and in convalescence these agglutinins dropped to an undetectable level. In Table II practically all subjects (about 90 per cent) examined in our work showed high serum levels of MCHA. The frequency with which MCHA was encountered in the current series has not been reported as occurring in either normal individuals or in patients suffering with illnesses other than viral hepatitis.[2, 3] The discrepancy in results obtained in different laboratories might be attributed to slight differences in technique used to demonstrate presence of the serum factor responsible for agglutination of monkey erythrocytes.

TABLE II. HEMAGGLUTINATION FINDINGS IN TESTS WITH CHICKEN AND MONKEY ERYTHROCYTES AND EXTRACTED AND UNEXTRACTED SERA OF NORMAL INDIVIDUALS

GROUP	SUBJECT	SPEC	SERUM TITERS OBTAINED WITH			
			CHICKEN ERYTHROCYTES		MONKEY ERYTHROCYTES	
			EXTRACTED SERUM	NONEXTRACTED SERUM	EXTRACTED SERUM	NONEXTRACTED SERUM
I	1	1*	0†	6	24	384
		2	0	6	12	96
	2	1	0	0	12	24
		2	0	12	6	6
	3	1	0	0	48	24
		2	0	0	96	96
	4	1	0	0	48	96
		2	0	0	12	96
	5	1	0	0	48	96
		2	0	6	6	96
	6	1	0	0	24	48
		2	0	0	24	48
	7	1	0	0	6	96
		2	0	0	0	24
	8	1	0	0	24	24
		2	0	0	192	96
	9	1	0	0	24	0
		2	0	0	12	0
	10	1	0	0	24	24
		2	0	6	12	24
	11	1	0	0	24	48
		2	0	0	6	12
	12	1	0	0	48	48
		2	0	0	12	48
	13	1	0	6	96	192
		2	0	6	24	192
	14	1	0	6	48	192
		2	0	6	48	192
	15	1	0	6	48	384
		2	0	6	384	96
	16	1	0	6	48	192
		2	0	12	48	192
	17	1	0	24	96	6
		2	0	12	96	12
II	18	1	6	6	24	192
		2	6	6	12	384
	19	1	6	12	48	768
		2	6	24	48	192
	20	1	12	24	48	96
		2	12	12	48	48

Numbers are reciprocals of serum titers.

*One month interval between first and second bleedings to obtain serum.

†0 = no pattern of agglutinated cells at 1:6 dilution, the lowest serum dilution tested.

Failure to Demonstrate in Sera From Convalescent Patients With Hepatitis Specific Inhibitor Useful in Development of Hemagglutination Inhibition Test.—When CCHA was first demonstrated to be present in sera of patients in the acute phase of hepatitis, it was hoped that a specific inhibitor directed against CCHA might be found in serum from patients in the convalescent phase. Such hopes were raised when the observation was made that presence of CCHA in sera of certain patients with hepatitis such as Patients 4, 7, and 10 in Table I was not detected by mixing unextracted serum with chicken erythrocytes, but activity of CCHA was manifested after extraction with acetone and ether. These observations suggested to us that there might be in sera of patients with hepatitis both CCHA and an inhibitor directed against it. This possibility is still under study, but up to the present time repeated attempts have failed to demonstrate in sera of convalescent patients with hepatitis an inhibitor directed against CCHA which might be employed in development of a specific serologic test for infectious hepatitis.

DISCUSSION AND SUMMARY

While serum reactions in tests to measure hemagglutinins reactive with chick erythrocytes may vary in individual patients, a pattern can be described as being somewhat characteristic for infectious hepatitis. The usual reactions observed in patients with infectious hepatitis are characterized by presence in acute illness of detectable serum levels of hemagglutinins reactive with chick red blood cells. In these cases in convalescence the chick cell hemagglutinin (CCHA) falls to a low or undetectable level or persists at a constant level. It is clearly evident, however, from the data presented in Table I, that about 1 in every 2 patients with hepatitis in our extensive series did not develop detectable CCHA. Monkey red blood cell hemagglutinin titers are high throughout the course of illness in patients with infectious hepatitis and, in tests as performed in our laboratory, do not differ markedly from titers found in normal individuals. When chick cell hemagglutinin is detected in patients with acute illness and this serum factor falls to a lower level in convalescence and the patient clearly does not have infectious mononucleosis, there is some justification in suspecting a case of infectious hepatitis. However, the chick erythrocyte hemagglutinin reaction is considered to be a nonspecific measure of serum alteration caused by presence of a factor about which only little is known. Results of preliminary work to delineate the physical and chemical properties of CCHA have been reported.[4, 6] Specific serologic diagnosis of infectious hepatitis will depend upon success of efforts to recover in an experimental host the infectious agent of this disease or to demonstrate in convalescent hepatitis serum a specific inhibitor directed against the hemagglutinin found in acute serum. In the absence of a specific test, the chicken erythrocyte agglutination procedure might prove to be a useful laboratory aid in the differential diagnosis of clinical jaundice when used as an adjunct to other tests to measure serum alteration accompanying liver damage. Its value, however, depends upon recognition of its limitations.

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THE ANTIBODY MOLECULE IN CHRONIC NONSPECIFIC THYROIDITIS: ELECTROPHORETIC PATTERNS OF SERUM

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IT IS now well established that chronic thyroiditis is associated with the presence of circulating autoantibodies.[1-7] Several reports have dealt with the attempt to identify the electrophoretic nature of this antibody by means of studies on the distribution of proteins in serum.[8-14] Conflicting claims have appeared concerning the interpretations. In general, the serum samples that have been chosen for study have been correlated with a clinical diagnosis for the presence of thyroiditis. The present report concerns an electrophoretic investigation of a number of sera which not only came from patients clinically diagnosed as having thyroiditis, but which were also tested serologically for the presence or absence of antibodies to human thyroid. A preliminary report has appeared.[15]

MATERIALS AND METHODS

Filter paper electrophoresis was conducted in the Spinco model R apparatus according to the manufacturer's suggested Procedure A. The stained (bromphenol blue) strips were examined in the analytrol unit of this system.

Free electrophoresis was conducted in the Spinco model H instrument after dialysis against barbital buffer of ionic strength 0.10, pH 8.6, and subsequent dilution to a protein concentration of about 1 per cent. Mobility calculations were used to ensure proper identification of every peak. Planimetric measurements of enlarged tracings provided the percentage composition. The results quoted are from descending channel measurements.

Protein concentration was determined in a Beckman DU spectrophotometer by the biuret method of Gornall and co-workers.[16] An extinction coefficient of 2.6 was determined for serum, as a result of comparison with dry weight on dialyzed samples.

The electrophoretic fractionations were conducted in a Spinco model CP continuous flow paper electrophoresis instrument in the cold room. The serum was diluted with an equal volume of barbital buffer, pH 8.6, ionic strength 0.02, and was run through at a low feed rate. Buffer was also running through at a slow rate. These rates were such that sample volumes of the order of 0.5 to 1 ml. were collected per hour.

The sedimentation studies were conducted in a Spinco model E ultracentrifuge. The customary methods of measurement and computation were employed.[17]

Serologic analyses on the sera and on serum fractions were performed by the tanned cell hemagglutination procedure, as previously described.[18] When a serum and its fractions were studied, all the titers were determined in the same experiment, with one exception.

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RESULTS

Normal Values.—The preliminary phases of this work showed that the elevations of globulin level to be expected, if any, would be slight or moderate.

TABLE I. NORMAL VALUES FROM FILTER PAPER ELECTROPHORESIS OF SERUM (48 SAMPLES)

COMPONENT	MEAN PERCENTAGE	STANDARD DEVIATION	NORMAL RANGE
Albumin	49.1	4.6_0	39.9-58.3
α_1-globulin	5.5	0.7_5	4.0- 7.0
α_2-globulin	12.2	0.7_5	10.7-13.7
β-globulin	15.3	1.3_5	12.6-18.0
γ-globulin	18.0	2.1_5	13.7-22.3

TABLE II. ELECTROPHORETIC DISTRIBUTION OF SERUM PROTEINS IN PATIENTS WITH CHRONIC THYROIDITIS

SERUM NO.	PATIENT	TOTAL PROTEIN (GM./DL.)	PERCENTAGE DISTRIBUTIONS					HEMAGGLUTINATION TITER
			γ	β	α_2	α_1	A	
1.	F. C.	—	**23.0***	**18.3**	**14.8**	6.6	**37.2**	9
2.	S. E.	8.5	**25.8**	10.9	11.1	3.7	47.7	6,400
3.	G. H.	9.4	**22.3**	17.4	10.7	6.2	43.3	5,000
4.	C. L.	8.0	20.1	12.1	**15.6**	**8.7**	43.6	700
5.	A. G.	7.3	20.2	14.9	14.9	**9.6**	40.4	9
6	M. R.	7.2	14.3	15.7	10.5	3.8	55.6	27
7.	M. R.	10.3	**29.0**	11.7	11.3	3.9	44.0	5,000
8.	M. B.	9.3	**27.0**	14.1	**15.2**	3.9	**39.8**	5,120
9.	R. B.	9.1	21.4	16.0	13.6	**10.2**	**37.6**	80
10.	K. L.	8.5	**23.2**	17.9	11.0	**7.6**	40.2	800
11.	J. B.	6.9	20.3	**24.5**	12.4	**9.5**	**33.2**	240
12.	M. Tr.	8.0	18.4	**20.4**	**13.9**	**9.2**	**37.9**	640
13.	M. Th.	7.7	18.0	**18.9**	**17.3**	7.0	**38.6**	2,000
14.	F. R.†	7.6	8.7, 9.0	10.0	9.5	3.8	59.0	5,000
15.	G. M.	9.7	**23.0**	**17.2**	10.2	3.5	46.1	700
16.	M. B.	8.8	10.1	10.5	**18.2**	4.0	**57.2**	700
17.	M. C.	8.2	15.1	13.9	**13.9**	3.5	53.7	240
18.	D. E.	10.4	16.2	14.5	10.6	5.6	53.1	3,200
19.	M. G.	9.3	15.7	**20.8**	10.6	4.6	48.5	900
20.	H. M.	8.8	16.2	10.2	**15.4**	4.3	53.8	81
21.	M. P.	5.9	18.9	13.5	**17.6**	5.4	44.6	27
22.	L. L.	8.8	21.0	16.7	10.3	4.3	47.7	9
23.	F. U.	10.1	**29.6**	14.8	11.8	4.4	**39.3**	240
24.	P. B.	6.2	16.9	16.3	**19.9**	**9.2**	**37.8**	27
25.	Y. G.	6.2	15.9	16.3	**16.3**	5.3	**46.1**	10
26.	W. G.	9.1	19.0	15.2	11.4	4.3	50.0	7,000
27.	M. V.	8.7	21.7	**19.3**	**14.0**	**7.2**	**37.6**	80
28.	C. K.	**6.7**	17.0	15.2	**15.8**	6.1	46.0	700
29.	A. P.	7.8	16.5	15.1	9.9	4.6	54.0	40
30.	J. J.	6.3	18.6	14.2	9.6	**7.1**	50.4	2,000
31.	C. P.	4.7	12.3	14.6	**14.6**	4.7	53.8	81
32.	C. V.	6.6	14.0	15.3	12.0	5.3	53.3	200
33.	E. M.	7.3	18.8	16.9	**15.0**	4.9	44.3	9
34.	M. H.	5.7	13.5	9.8	9.8	4.9	**34.9**	81
35.	E. W.	7.7	**27.9**	13.0	**14.3**	5.8	**38.9**	700
36.	W. B.	3.6	**23.5**	15.7	**14.8**	**7.8**	**38.2**	64
37.	B. L.	5.5	20.7	16.1	8.8	5.5	48.8	700
Number of values outside normal range			10	6	17	10	12	
Per cent of values outside normal range			27	16	46	27	32	

*Values in bold face are outside normal range.

†The γ-globulin appeared to show two components—8.7 per cent of γ_2-globulin and 9.0 per cent of γ_1-globulin.

It was thus realized that the normal values against which comparison would be made must be carefully established. Normal sera were obtained from 48 apparently healthy sophomore dental students. These sera were studied by filter paper electrophoresis, and the mean value, $\bar{x}$, for each of the 5 components was computed along with the standard deviation, σ. These results, along with each corresponding range, are shown in Table I. The normal range was chosen as that for 2 standard deviations on either side of the mean. This would include 95.5 per cent of values in a normal distribution. Since one is only interested in elevation of values (or depression for albumin), one would expect, on the average, only about 2 per cent of normal values to be outside each range chosen.

TABLE III. REPRODUCIBILITY OF EXAMINATION BY FILTER PAPER ELECTROPHORESIS

SERUM	RUN NO.	PERCENTAGE DISTRIBUTION				
		γ	β	α_2	α_1	A
S. E.	11	26.8	13.3	10.0	5.8	44.0
	23	25.1	11.1	12.8	2.2	48.8
	25	25.4	8.2	10.6	3.2	52.5
Average		25.8	10.9	11.1	3.7	48.4

Chronic Thyroiditis and Struma Lymphomatosa.—A study was made with the sera from 37 patients having the clinical diagnosis of thyroiditis. All of these samples had been shown to contain circulating antibodies to human thyroid extract. Whereas only 10 of them showed precipitins (by the Oudin test), all of them showed positive results by tanned cell hemagglutination. They were examined by filter paper electrophoresis with the results shown in Table II. It must be concluded that only 27 per cent of the samples could be considered to have elevated γ-globulin. On the other hand, it can be seen that 46 per cent of the cases showed an increase in α_2-globulin. It should be noted that none of the elevations are very large. Nonetheless, there are lesser, but apparently significant, occurrences of increased β- and α_1-globulin levels. To ensure further the reliability of filter paper electrophoretic methods, some additional repeated runs were done. A typical series on the serum with elevated γ-globulin (from Patient S. E.) is shown in Table III (the values in Table II are the averages). It is clear that despite slight variations, comparison with regard to the normal values can be safely made.

TABLE IV. SERUM DISTRIBUTIONS ACCORDING TO OPTICAL ELECTROPHORESIS

SERUM	PERCENTAGE DISTRIBUTION					
	γ_2	γ_1	β	α_2	α_1	A
F. R.	10.5	8.2	12.7	9.6	4.5	54.6
S. E.	23.7	—	20.0	6.8	4.2	45.4

One of the sera (from Patient F. R.) seemed to exhibit a prominent additional component, apparently γ_1-globulin. To confirm this finding, it was examined by means of optical electrophoresis. The other serum (from Patient S. E.) was also checked by this means. The data, shown in Table IV, adequately substantiate the filter paper findings. The actual patterns are shown in Fig. 1. The serum from Patient F. R. was also examined in the analytical

ultracentrifuge. It showed 80.4 per cent of the 4.5 S-component, 16.4 per cent of 7 S, and 3.0 per cent of 18 S. These values are within the normal ranges.

It was of special interest to study serum patterns in those patients with a histologic diagnosis of struma lymphomatosa, or Hashimoto's disease, according

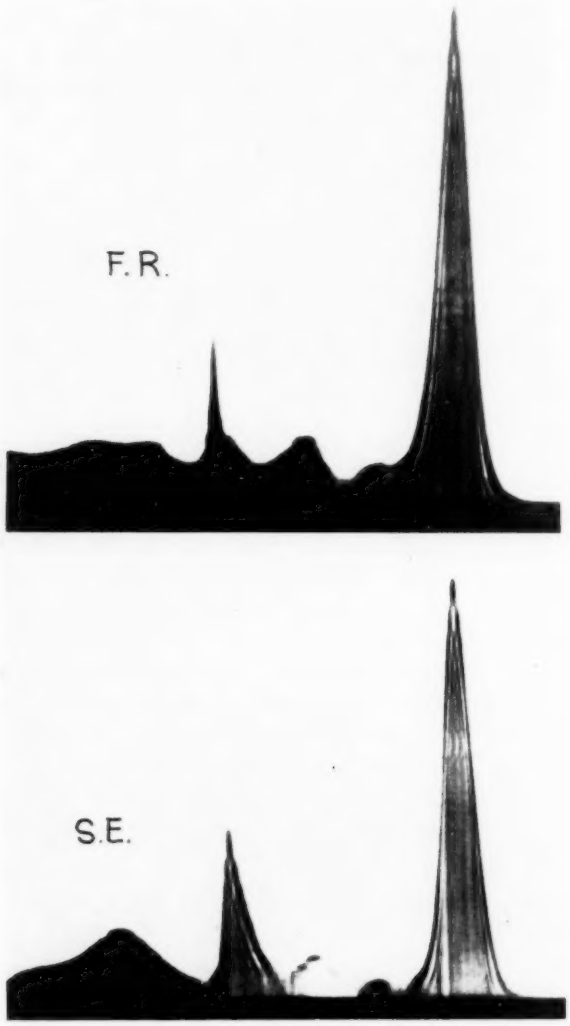

Fig. 1.—Electrophoretic patterns of serum from patients with thyroiditis. Barbital buffer: pH 8.6, $\Gamma/2 = 0.10$. Electric field strength: 6.9 volts per centimeter. Time: 110 minutes. Upper: Serum 14 of Table II (Patient F. R.) at a total protein concentration of 0.7 per cent. Schlieren angle: 40 degrees. Lower: Serum 2 of Table II (Patient S. E.) at a total protein concentration of 0.5 per cent. Schlieren angle: 50 degrees.

to the strictest definition of the term. The (histologic) criteria for distinguishing Hashimoto's disease from chronic thyroiditis, as used by our colleague, Dr. Terplan, Professor of Pathology, have been described in a previous article [9]

These cases were consistently negative when tested for autoantibodies.* The same 14 sera that were described in that study were now investigated by means of filter paper electrophoresis. The results are shown in Table V. Although demonstrable antibody is absent, the γ-globulin level is elevated in 57 per cent of the cases. Along with this, there is again a substantial incidence of elevated α_2-globulin. In contrast, *none* of them show an increase in β- or α_1-globulin or a decrease in albumin.

Electrophoretic Fractionation of Thyroiditis Sera.—

Serum from Patient F. R.: Since a sufficient quantity of this serum (Serum 14 in Table II) was available, and since it had shown a uniquely elevated γ_1-globulin, it was decided to attempt to fractionate it by means of continuous flow paper electrophoresis in the hope of localizing the antibody in one of the globulin fractions. This was carried out at 1,000 volts and 70 Ma. for 4 hours in the cold room on a sample of 10 ml. of serum diluted with an equal volume of buffer, and using a continuous flow of the barbital buffer, pH 8.6, ionic

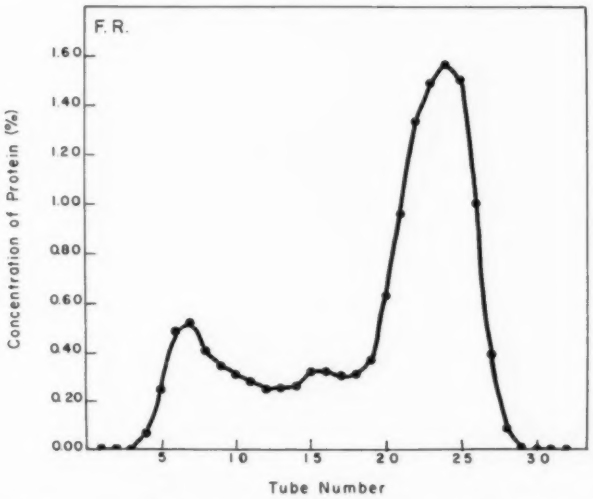

Fig. 2.—Protein concentration plotted against fraction number from continuous flow paper electrophoresis of serum from Patient F. R. (Serum 14, Table II).

strength 0.02. Each of the resultant 32 fractions was analyzed for protein concentration by means of the biuret method. The graph of protein concentration versus fraction number is shown in Fig. 2. Each fraction was then examined by filter paper strip electrophoresis. In doing this, each group of 8 strips included 1 for the whole serum, in order to ensure greater reliability of identification. It was seldom possible to obtain only a single component in any one fraction. The sample size applied for each fraction was varied in accordance with the protein content, so that a sensitivity would be realized which was of the same order as in the standard procedure for whole serum.

*Subsequent to the report on the first group of 14 such sera, 11 more have been studied, of which 2 showed circulating autoantibodies (hemagglutination titer of 6,400 in each).

In accordance with these results, the fractions were combined into 7 pools. These pools were made more concentrated by means of dialysis for several hours, in the cold, against a 30 per cent solution (previously dialyzed against 0.15M sodium chloride) of polyvinylpyrrolidone (PVP); they were then measured for protein concentration, and again analyzed by filter paper electrophoresis. These pools were also studied for their antibody content by means of tanned cell hemagglutination. The actual filter paper strips are shown in Fig. 3. The analyses are shown in Table VI. It can be seen that the *antibody* titer is very high in Pools 1 and 2, and *only* in these pools, while the *protein* levels of all the pools are of similar magnitude.

TABLE V. ELECTROPHORETIC DISTRIBUTION OF SERUM PROTEINS IN PATIENTS WITH STRUMA LYMPHOMATOSA (HASHIMOTO)

SERUM NO. PATIENT	TOTAL PROTEIN (GM./DL.)	PERCENTAGE DISTRIBUTION				
		γ	β	α_2	α_1	A
1. R. L.	7.5	**24.4***	12.4	9.1	3.6	50.6
2. N. S.	11.1	**23.8**	17.4	11.6	4.6	42.4
3. C. O.	8.5	16.0	9.4	**14.2**	6.6	53.8
4. B. S.	8.8	**22.4**	16.9	12.0	4.0	44.8
5. L. S.	9.1	**23.4**	15.8	10.8	5.0	45.0
6. G. R.	9.2	**23.8**	12.5	**13.9**	5.4	44.4
7. G. N.	8.6	20.8	16.9	8.5	6.2	47.6
8. I. M.	8.6	16.6	14.0	**16.0**	5.6	47.8
9. F. M.	8.4	19.0	16.1	12.7	4.4	47.8
10. H. F.	9.3	16.9	16.3	10.7	3.9	52.3
11. D. D.	8.2	**25.0**	11.3	9.4	4.6	49.2
12. A. C.	9.9	**23.0**	7.1	9.4	2.9	57.7
13. B. C.	10.0	16.6	11.7	**14.9**	6.0	50.8
14. V. P.	10.2	**23.6**	10.4	**16.9**	6.2	42.9
Number of values outside of normal range		8	0	5	0	0
Per cent of values outside of normal range		57	0	36	0	0

*Values in bold face are outside normal range.

TABLE VI. ELECTROPHORETIC AND IMMUNOLOGIC ANALYSIS OF FRACTIONS PREPARED FROM SERUM FROM PATIENT F. R.

POOL	FRACTIONS	COMPONENTS	PROTEIN CONCENTRATION (%)	HEMAGGLUTINATION TITER
1	4-9	$\gamma_2+\gamma_1$	0.91	> 15,000
2	10-11	$\gamma_2+\gamma_1$(85%), β(15%)	1.87	> 15,000
3	12-15	γ_1(30%), β(70%)	1.53	625
4	16-17	β(42%), α_2(58%)	3.40	125
5	18	α_2(92%), α_1(8%)	2.16	25
6	19-23	α_2(22%), α_1(7%), A(71%)	3.12	5
7	24-25	A	6.47	5
Whole Serum		—	7.55	5,000*

*The whole serum was not examined at the same time that the fractions in this study were examined.

Serum from Patient M. Th.: Since the serum analyzed first (from Patient F. R.) was one with an elevated γ-globulin (γ_1-globulin), it was of interest to study a serum which had instead a rise in the α_2-component. Serum from Patient M. Th. (Serum 13 in Table II) was therefore separated in similar manner.

The biuret graph is shown in Fig. 4. Pools of fractions were formed and concentrated, in the same manner as before. These 6 pools were re-examined electrophoretically and immunologically. The filter paper strips are shown in Fig. 5. Since so little material was available, except for the albumin pool, protein concentration was measured on only the albumin pool. The concentrations for the others were then estimated from the results of the electrophoresis by comparison of the total area scanned for each of the other pools compared to that for the albumin pool. Although it is difficult to apply to the paper strips just the same sample size for all pools, the accuracy can be considered adequate for the purpose at hand. The analytical results are shown in Table VII. Again

TABLE VII. ELECTROPHORETIC AND IMMUNOLOGIC ANALYSIS OF FRACTIONS PREPARED FROM SERUM FROM PATIENT M. TH.

POOL	FRACTIONS	COMPONENTS	PROTEIN CONCENTRATION (%)	HEMAGGLUTINATION TITER
1	4-9	γ	0.32	> 15,000
2	11-13	β	0.86	625
3	15-16	β(80%), α_2(20%)	1.01	625
4	17-19	α_2	1.02	125
5	20-22	α_2(40%), α_1(13%), A(47%)	2.05	negative
6	23-26	A	2.43	negative
Whole Serum		—	7.70	2,000

TABLE VIII. ELECTROPHORETIC AND IMMUNOLOGIC ANALYSIS OF FRACTIONS PREPARED FROM SERUM FROM PATIENT M. TR.

POOL	FRACTIONS	COMPONENTS	PROTEIN CONCENTRATION (%)	HEMAGGLUTINATION TITER
1	4-9	γ	0.31	625
2	10-14	γ (75%), β (25%)	0.24	625
3	15-18	β	0.58	25
4	20-24	α_2 (46%), α_1 (9%), A (45%)	0.65	negative
Whole Serum		—	8.01	625

TABLE IX. ELECTROPHORETIC DISTRIBUTION OF COMPONENTS IN THYROIDITIS SERUM (FROM PATIENT F. R.) BEFORE AND AFTER ABSORPTION WITH THE OPTIMAL QUANTITY OF THYROID EXTRACT

PATTERN	SERUM	PERCENTAGE DISTRIBUTION					
		γ_2	γ_1	β	α_2	α_1	A
Descending	Unabsorbed	11.1	8.7	14.4	10.9	4.5	50.5
Descending	Absorbed	9.2	7.7	12.1	13.8	6.3	50.8
Ascending	Unabsorbed	9.6	6.9	12.5	9.7	4.7	56.5
Ascending	Absorbed	7.2	6.9	9.4	12.9	6.3	57.3

the γ-globulin titer is considerably higher than the others, even though its protein level is lower. Certainly, the α_2-globulin cannot be implicated in the hemagglutinating antibody activity.

Serum from Patient M. Tr.: Another serum (Serum 12 in Table II) with elevated α_2-globulin was available, and it too was investigated in this same way. The biuret graph is shown in Fig. 6, and the filter paper strips for the pooled

fractions are shown in Fig. 7. The analytical data are given in Table VIII. The results, although less striking, are of the same nature as for the other sera.

Absorption of Antibody by Specific Antigen.—One of these sera (from Patient F. R.) was also studied by quantitative precipitin analysis. The optimal quantity of antigen (crude extract of thyroid) was thus determined, and this maximally absorbed serum was examined by optical electrophoresis. A sample of unabsorbed serum was again examined at this time for direct comparison. A more detailed description of this kind of investigation will be given in another paper[20] in connection with a later study. The distribution of components,

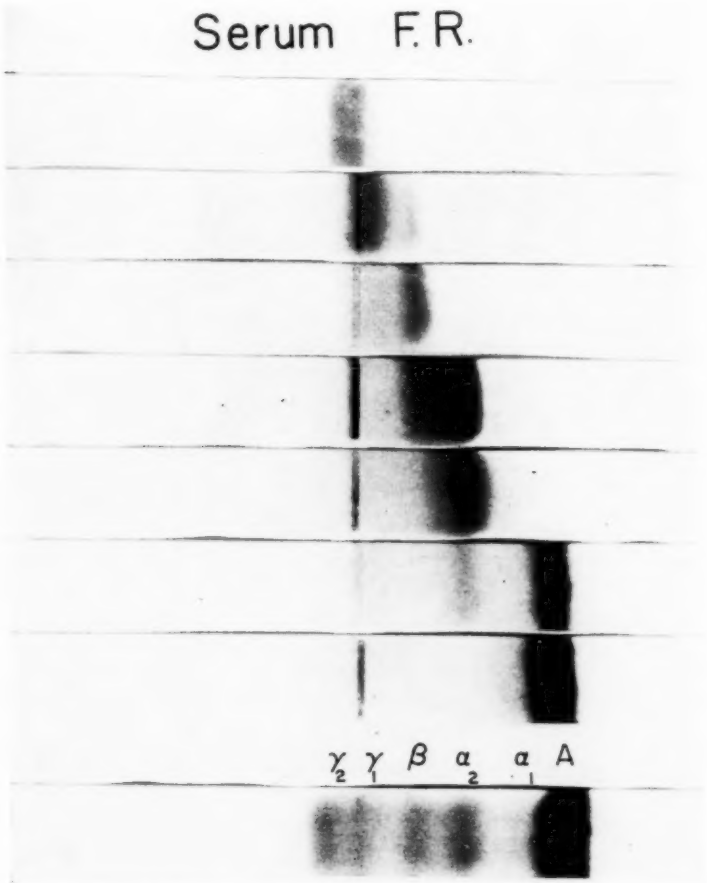

Fig. 3.—Paper strips from electrophoresis of pooled fractions from serum from Patient F. R. Pools are numbered downward; bottom strip contains sample of original whole serum.

before and after absorption, is shown in Table IX. It can be seen that if any component is diminished by absorption, it is γ_2-globulin (or β-globulin) *not*, as one might have expected, γ_1-globulin. This is especially clear from the ascending patterns. However, any apparent changes are so small that they may be

vitiated by the experimental error involved in these area measurements. The one reliable observation is that an identification of antibody with γ_1-globulin is not established, although it is just this component that exhibits a marked elevation in the original serum.

DISCUSSION

This investigation has shown that in only 27 per cent of a group of serologically positive sera was a significantly elevated level of γ-globulin seen. In contrast, 46 per cent showed increased α_2-globulin and 27 per cent showed increases in α_1-globulin. Even increases in β-globulin occurred in 16 per cent of cases. Only 4 sera (11 per cent) showed simultaneous elevations in both γ- and α_2-globulins. It is clear that these statistics cannot be used as a sure basis for predicting the presence of antibody or for establishing the electrophoretic identity of any antibody present. This becomes especially obvious when it is found that in the group of serologically negative sera an even higher incidence was seen of elevated γ-globulin—the component that had been suspected of harboring the antibody. Clearly, such serum analyses are of no real clinical value, either, as has been emphasized by others.[11]

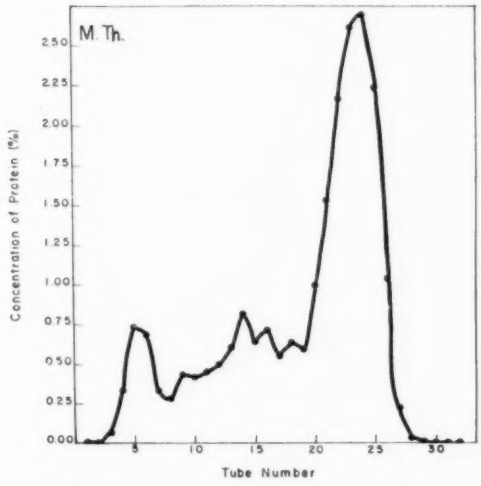

FIG. 4.—Protein concentration plotted against fraction number from continuous flow paper electrophoresis of serum from Patient M. Th. (Serum 13, Table II).

It is also very difficult to establish any correlation between globulin elevation and antibody titer. One may take, arbitrarily, a titer of 700 as a demarcation. Of the 37 sera, 20 have titers below 700, and of these, 3 (15 per cent) have elevated γ-globulin; 17 have titers of 700 or higher, and of these, 7 (41 per cent) have elevated γ-globulin. These would be compared with 27 per cent for the entire group. While it is quite possible that the incidence of γ-elevation increases with antibody titer, it must be noted that the other globulins seem to behave in an opposite manner. For example, the α_2-elevation occurs in 55 per

cent of the sera with titers < 700, but only in 35 per cent of the sera with titers ≥ 700, compared to 46 per cent for the whole group. The relationship is probably a more complicated matter.

These findings on sera from patients with chronic nonspecific thyroiditis find some concurrence in reports from other laboratories. Unfortunately, comparison is often difficult for several possible reasons: (1) the diagnosis or its criteria are inadequately described; this is especially important if the study does not include serologic descriptions; (2) the ranges of normal values are not provided for the electrophoretic patterns, or if provided, there is no mention of

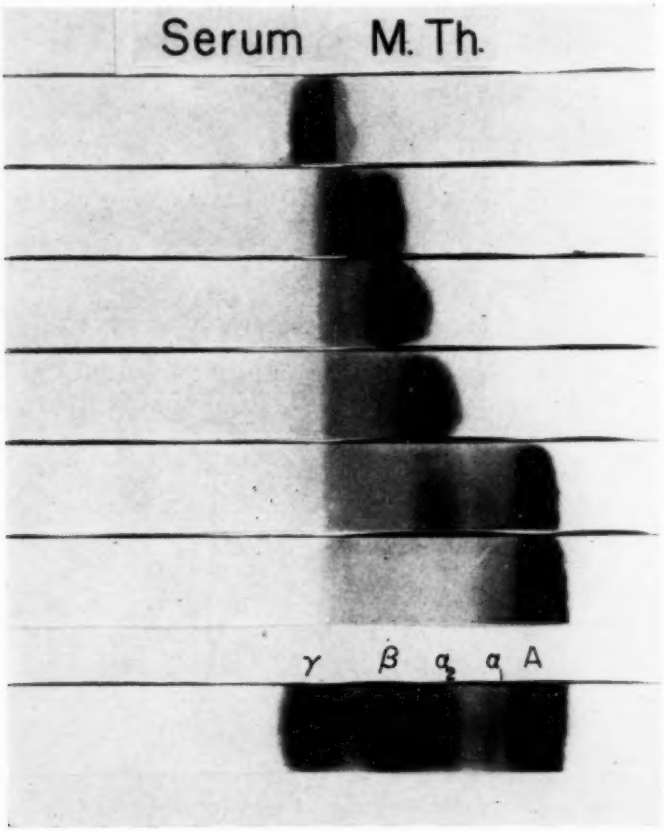

Fig. 5.—Paper strips from electrophoresis of pooled fractions from serum from Patient M. Th. Pools are numbered downward; bottom strip contains sample of original whole serum.

the number of standard deviations employed as criterion; and (3) the number of sera studied is inadequate for statistical reliability. Nonetheless, some comparisons can be attempted. The following studies on subacute thyroiditis are of interest. Skillern and co-workers[11] used optical electrophoresis in an extensive investigation and found that 2 of 6 sera showed slight elevation of γ-globulin, 1 showed slight elevation of β-, and 1 of α_1- and α_2-globulin. They described the

patterns as essentially normal. While the normal ranges are given, there is no description of the standard deviation. This group's contrasting study on struma lymphomatosa will be mentioned below. Earlier reports include Fromm and associates[5] and Werner.[12] Each of these described 1 patient with increased level of γ-globulin. However, in the former, α- and β-globulin levels were also high; in the latter, the α-globulin level was also high. Stemmermann,[13] on the other hand, using paper electrophoresis, claims that α_2 was high in 4 cases. This is of interest, since the most common change reported in the present study is the increase in α_2. More recently, Weissman and Perlmutter[14] have objected to the use of paper electrophoresis and to the conclusion that α_2-globulin is uniquely affected. They studied 8 sera by optical electrophoresis and concluded that γ was increased in all of them, while 7 of 8 showed increased α_1 and α_2. While it is of great interest that several components are implicated, it must be noted that no normal ranges are given in this report; the pathologic values are merely compared to the mean normal values.

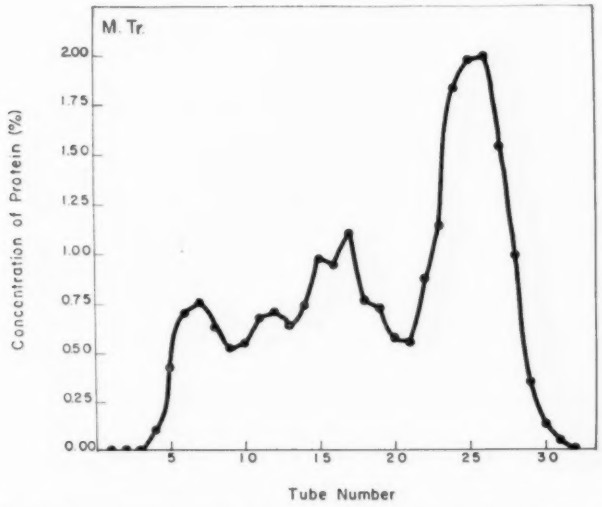

Fig. 6.—Protein concentration plotted against fraction number from continuous flow paper electrophoresis of serum from Patient M. Tr. (Serum 12, Table II).

None of the above citations include any serologic studies as to antibody content. The first studies to include this criterion appear to have been those of Doniach and associates.[3, 4] They reported the presence of specific thyroid autoantibodies in 11 patients with Hashimoto's disease (or lymphadenoid goiter).* They also found, by paper electrophoresis, that the γ-globulin was elevated in all these sera. They provide means and ranges for a group of normal sera, although they do not give standard deviations. They found, however, that none of the other globulins are elevated. Similar findings have again been reported more recently.[7, 21] This absence of elevations in other globulins would

*These workers do not differentiate between chronic nonspecific thyroiditis and struma lymphomatosa, in the sense of previous reports from this Laboratory.[19]

be in disagreement with all the reports mentioned above (which deal with subacute thyroiditis) and with the present report (which deals with antibody-containing sera).

More recently, some other studies have also referred to these questions. Blizzard and co-workers[22] studied 161 patients with thyroid disease, and found 54 to be positive for antibody. Their major interest was to correlate the immunologic findings with the clinical descriptions. They mention, however, that serum globulin determinations on 82 sera from patients with thyroid disease (46 with antibodies) showed that those with antibodies "tended to have higher globulin levels." They also state that there is not sufficient difference to permit correlation of globulin level to the presence of antibodies. While there were

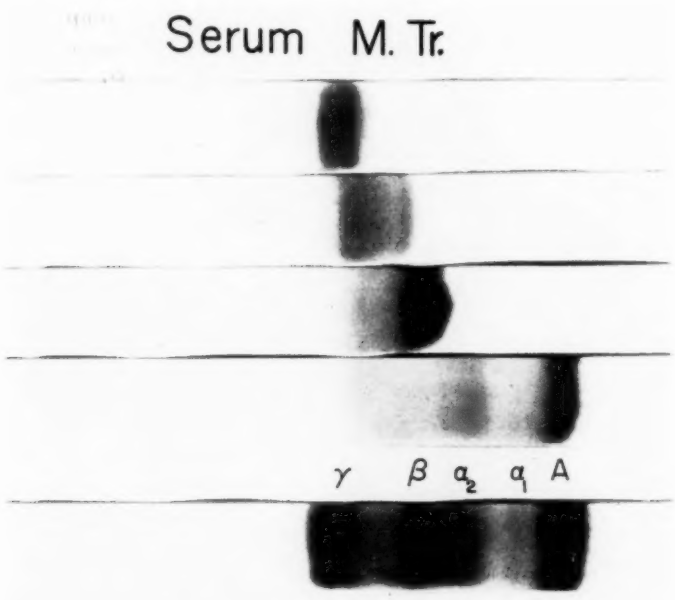

Fig. 7.—Paper strips from electrophoresis of pooled fractions from Serum 12 (Table II). Pools are numbered downward; bottom strip contains sample of original whole serum.

no electrophoretic analyses, the fact that only some of the positive sera showed higher levels of total globulin than did negative sera is in essential agreement with the data presently reported. The report of Cline and associates[23] is also a correlation of presence of antibody with the clinical description of the thyroid disorder. In one case, a serum fractionation was performed and the antibody was localized in the γ-globulin fraction, although no details are provided.

In addition to this body of reports, the extensive clinical study by Skillern and co-workers[9] is highly instructive. This investigation deals with 25 patients with struma lymphomatosa. The contention is set forth that this condition is not a true thyroiditis, but rather an idiopathic primary failure of the thyroid cells followed by secondary changes resembling thyroiditis. There were

no serologic data. By means of optical electrophoresis on 20 of these sera, 16 were found to have elevated γ-globulin, although one of these is just borderline. However, this result is in remarkable agreement with the findings in our report in showing a high incidence of γ elevation. Further agreement is found in the low proportion of α elevation—they found only 1 of 20 (α_1 and α_2 were not resolved). Some slight disagreement seems to occur as to the β level; they found 5 of 20 to be elevated. It should also be mentioned that Fromm and associates[8] reported an elevated γ-globulin level in 4 sera from patients with struma lymphomatosa.

Returning to the data presented in this study, it is clear that γ-globulin *cannot* be implicated as the antibody-containing fraction directly from the patterns of protein distribution. In the group of serologically negative sera, the incidence of γ-globulin elevation was considerably higher than among the sera that contained circulating antibodies. In the group of serologically positive sera, furthermore, the level of α_2-globulin is much more often increased than is γ-globulin, suggesting two possibilities. Either the antibody is an α_2-globulin (as well as a γ-globulin, perhaps), or these abnormal protein distributions are reflections of disturbances in protein metabolism and do not *primarily* reflect the immune state. To settle the question unequivocally, fractionation by continuous paper electrophoresis was utilized. The first serum to be studied in this manner (from Patient F. R.) was unique in that not one of the customary components was elevated; the increased level was instead for γ_1-globulin. Table VI shows that γ-globulin contains the antibody, whereas the other globulins do not. The data on Pool 3 are especially interesting. Although γ_1-globulin is approximately as prominent in this fraction as it is in Pool 2, the antibody titer has fallen by more than twenty-five-fold. This is strong evidence that the antibody is not only γ-globulin, but is in fact γ_2-globulin. Further support for this conclusion is suggested by the absorption-electrophoresis study described for this same serum (Table IX). It should then be carefully noted that here again one cannot correlate serum globulin levels with antibody content.

Because of the possibility that α_2-globulin might be associated with the antibody, the 2 other sera were fractionated. Each of these initially showed an elevated α_2 level and no elevated γ level. The results in Table VII and VIII are quite conclusive in again identifying the antibody as a γ-globulin. It should also be noted that all 3 of these sera showed precipitin activity.

Other reports have appeared on fractionation of the globulin components. Studies have already appeared from our Laboratory which indicate that the hemagglutinating antibody is a γ-globulin, as shown in one human serum by means of starch electrophoresis combined with tanned cell hemagglutination.[24] Earlier studies with rabbit thyroid autoantibody had similarly indicated an identification with γ-globulin.[25] Roitt and co-workers[26] make brief mention of zone electrophoresis on cellulose columns, and state that no clear qualitative differences could be detected between the γ-globulins from patients with Hashimoto's disease and from normal serum. Only liver function flocculation tests are mentioned. Doniach and Roitt[7] refer briefly to fractionation of serum from

patients with Hashimoto's disease by zone electrophoresis, and state that the antibody is "located exclusively in the gamma globulins," as tested by precipitation. Roitt and Doniach[21] briefly describe a similar separation on filter paper, followed by elution. They state that only the γ-globulin fraction gave positive complement fixation with thyrotoxic thyroid extracts. Cline and associates[23] refer to one serologically positive serum which was fractionated by zone electrophoresis on cellulose columns, and state that the antibody was localized in the γ-globulin fraction. In a more recent and much more extensive study, Roitt and co-workers[27] utilized zone electrophoresis and also immunoelectrophoresis, and presented convincing evidence for the γ-globulin nature of this antibody, using the serologic criterion of visible precipitation. Whether different molecular entities are involved as precipitins or agglutinins may remain a question to be explored further.

SUMMARY

1. Serum from humans with chronic nonspecific thyroiditis showed some abnormalities of protein distribution. Of 37 sera, all proved serologically to contain thyroid antibody; the following incidence of elevated (beyond 2 standard deviations) levels occurred: 10 (27 per cent) for γ-globulin, 6 (16 per cent) for β-globulin, 17 (46 per cent) for α_2-globulin, and 10 (27 per cent) for α_1-globulin. One sample showed an increase in γ_1-globulin. Albumin was lowered in 12 (32 per cent) of the sera.

2. Serum from humans with struma lymphomatosa also showed some peculiarities of electrophoretic distribution, although all were serologically negative. Of 14 sera, 8 (57 per cent) were elevated in γ-globulin, whereas 5 (36 per cent) were increased in α_2-globulin. No abnormality was found in β- or α_1-globulin or albumin.

3. Three sera were fractionated by means of continuous flow paper electrophoresis. One had exhibited increase in γ_1-globulin; the other 2 in α_2-globulin. In all 3 sera, the antibody, as judged by tanned cell hemagglutination, was identified as a γ_2-globulin.

We would like to acknowledge the skillful technical assistance provided in these studies by Mrs. Ellen Chiazze.

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EVALUATION OF BIOASSAY FOR URINARY CATECHOL AMINES WITH STRIPS OF RABBIT AORTA

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PHEOCHROMOCYTOMA, a chromaffin cell tumor which secretes norepinephrine and epinephrine, arises most commonly in the adrenal medulla, and is said to be responsible for approximately one half of one per cent of all cases of hypertension.[1-4] The tumor was first described in 1886 by Fraenkel[5] and was again described in 1892, by Berdez.[6] In 1922 the Labbé syndrome of intermittent hypertension was associated with paraganglioma, including pheochromocytoma.[7] Since then the clinical picture has become well established.[8, 9]

In 1946 the injection of histamine became the first specific diagnostic procedure for pheochromocytoma.[10] Histamine is one of several compounds which may provoke secretion of catechol amines stored by the tumor, thereby reproducing the paroxysmal hypertension and other characteristic signs and symptoms.[11] In 1952 phentolamine (Regitine) was used to block the pressor action of catechol amines and thus temporarily reduce the hypertension associated with pheochromocytoma.[12]

In 1950 a more direct approach to the diagnosis of pheochromocytoma became possible when von Euler[13] devised a chemical method of estimating the small amounts of catechol amines which occur in body fluids. The method was fluorometric and required prior chromatographic separation of the amines. Although simplifications and improvements of the chemical assay have been described, the technique remains cumbersome; moreover it is not specific for pressor catechol amines.[14, 15] Chemically related but pharmacologically inert compounds like 3,4-dihydroxyphenylacetic acid as well as unrelated compounds like bilirubin may interfere with the determination.[14, 15]

Some circulating catechol amine is renally excreted, as demonstrated by the recovery of 1 to 5 per cent of injected norepinephrine and epinephrine from urine.[16] The urinary concentration of such amines, therefore, reflects their rate of production. The normal excretion of catechol amines varies from 50 to 100 γ per day, whereas a patient with a pheochromocytoma ordinarily excretes over

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300 γ per day.[17] In 1955, Helmer[18] reported a simple bioassay for urinary catechol amines. He adapted the technique that Furchgott[19] had been using to study the pharmacology of epinephrine and other vasoactive drugs. Pressor substances were estimated by their effect on spirally cut strips of rabbit aorta. Like the chemical method, this bioassay lacks specificity since other vasoactive drugs may constrict the aortic strip. Specificity can be increased by demonstrating that pressor activity is enhanced by cocaine and blocked by Dibenamine. Pressor, but noncatechol, amines like 3-hydroxytyramine are not enhanced by cocaine, and angiotensin is not inhibited by Dibenamine. In our hands a major advantage of the bioassay has been its simplicity. Our purpose here is to evaluate its efficacy as a diagnostic procedure. We have no experience with other bioassays such as those which make use of the whole animal.[20, 21]

METHOD

Circumferential muscle fibers of rabbit aorta can be made to contract in proportion to the concentration of catechol amines in a surrounding bath. In order to prepare the test system, a large normal unanesthetized female rabbit was killed by a blow upon the head and was then bled. Its descending thoracic aorta was quickly removed and placed in a Petri dish which contained Kreb's Ringer bicarbonate solution* at room temperature. After excess fat and connective tissue were removed, strips approximately 2 mm. wide and 25 mm. long were cut in a spiral manner. Each strip consisted of about 2 turns with the circumferential muscle fibers making an angle of about 15 degrees with the long axis of the strip. A strip was then mounted in an individual chamber so that it was completely bathed by 20 ml. of Kreb's Ringer bicarbonate solution, with a pH of 7.4 and at a temperature of 37° C., to which had been added 40 mg. of glucose and 0.067 mg. of disodium calcium ethylenediamine tetraacetate and through which 100 ml. per minute of 95 per cent oxygen and 5 per cent carbon dioxide was continually bubbled. Exactly 20 mm. separated the points at which the bottom of the strip was fixed and the top was attached to the lever of an ink-writing, gravity-type pen which gave a fourfold amplification of contractions. The lever was counter weighted so that a constant tension of 1.7 grams was exerted on the strip. After a strip had been allowed 2 hours for complete relaxation, calibration was accomplished by the addition of 2 γ of norepinephrine to the bath with a resultant shortening of the strip of approximately 30 per cent in 5 minutes. The bath containing the amine was then removed and the strip was washed immediately and again after 5 minutes with fresh Kreb's Ringer bicarbonate solution. Within 15 minutes the strip had completely relaxed and regained its previous responsiveness.

For each assay, 0.04 ml. of urine was added to the 20 ml. bath which surrounded the strip. Aliquots of single, rather than 24 hour, specimens were assayed. In order to minimize destruction of catechol amines, immediately after being voided all specimens were acidified to a pH between 2.5 and 3.0 with 6N hydrochloric acid and were then stored at 4° C. until assay. If 0.04 ml. of urine produced less than one-tenth as much shortening as did 2 γ of norepinephrine, an additional 0.20 ml. was added without changing the bath. An attempt was made to enhance any significant contraction of the strip by the addition of 20 γ cocaine to the bath. If cocaine did enhance contraction, the strip was washed and allowed to relax. Then 2 γ of Dibenamine and, after 5 minutes, the previously effective volume of urine were added to determine whether the pressor effect was inhibited. In the event that cocaine enhanced and Dibenamine inhibited a response, the amount of pressor material was estimated

*The Kreb's Ringer bicarbonate solution was composed of 100 parts 0.90 per cent (by weight) NaCl, 4 parts 1.15 per cent KCl, 3 parts 1.22 per cent $CaCl_2$, 1 part 2.11 per cent KH_2PO_4, 1 part 3.82 per cent $MgSO_4 \cdot 7H_2O$, and 21 parts 1.30 per cent $NaHCO_3$, all dissolved in deionized distilled water.

from the percentage shortening of the strip. Approximately 3 per cent shortening was produced by 0.03 γ of norepinephrine in the 20 ml. bath which surrounded the strip. One liter of urine containing 750 γ of norepinephrine would have 0.03 γ per 0.04 ml. and, hence, would produce an easily measurable contraction. When 0.04 ml. of urine proved inert, the sensitivity of the assay was increased sixfold by the addition of 0.2 ml. of urine. All results have been expressed in terms of norepinephrine, without any further demonstration that the pressor material was catechol amine.

MATERIAL

During the 30 months beginning with January, 1956, a strip of rabbit aorta was used in our laboratory 217 times to assay the concentration of urinary catechol amines for 147 hypertensive patients in whom the diagnosis of pheochromocytoma was considered. Of these patients, 123 were from hospitals, and 24 were outpatients. Their average age was 48.4 years with a range of from 2 to 76 years. Sixty-nine of them were male and 23 were Negro.

Fig. 1.—Chest x-ray prior to surgical procedure for the 30-month-old patient of Case Report 1.

A history of hypertension, diabetes, and hyperthyroidism was sought from each patient and each was questioned as to headache, tachycardia, and sweating. Physical findings including pulse rate, blood pressure, cardiac size, and abdominal masses were recorded. Cardiac and renal function were evaluated in all patients, and thyroid and pancreatic function in some. Each patient had an electrocardiogram and roentgenogram of the chest. Each had at least 1 urinalysis and an estimation of circulating nonprotein nitrogen; for 82 a concentration-dilution test was performed and phenol red excretion or creatinine clearance was

measured. The level of fasting blood glucose was measured for 129 patients, and basal metabolism or protein-bound iodine was determined for 74. Two patients had benzodioxane and 69 had phentolamine tests; however, 45 of the latter had received sedatives or other drugs which might have interfered with interpretation of the results. Histamine or other provocative tests were performed for 14 patients. Eighty-two patients had roentgenographic studies which included either pyelography or perirenal air insufflation. The adrenal areas of 19 patients were surgically explored.

Case Report 1.—A 30-month-old Negro girl entered St. Louis Children's Hospital because of frequent episodes of severe sweating accompanied by a generalized rash occurring over a period of 10 months. On one occasion a systolic pressure of 190 mm. Hg and a pulse rate of 140 beats per minute had been observed. On admission the child had an erythematous papular rash over the chest, slight cervical adenopathy, and a blood pressure which varied from 160/90 to 170/100 mm. Hg in the sitting position and which fell to 130/70 mm. Hg on standing. Three per cent of the circulating leukocytes were eosinophiles. There was a trace of proteinuria and minimal microscopic hematuria. Intravenous pyelograms were normal, but a roentgenogram of the chest (Fig. 1) revealed a paravertebral calcified mass in the upper lobe of the left lung. No evidence of metastasis was found. Two bioassays for catechol amine revealed the pressor equivalent of 0.8 and 0.6 γ of norepinephrine per milliliter of urine.

During the first week of hospitalization the patient's diastolic pressure was consistently above 100 mm. Hg. Frequent episodes of profuse sweating accompanied by a blood pressure of 190/120 mm. Hg and a pulse of 140 beats per minute were observed. On the seventh day 12.5 mg. of phentolamine was administered orally. Within 15 minutes the blood pressure fell from 190/120 to 90/60 mm. Hg where it remained for 2 hours. For the next 5 days 10 mg. of phentolamine administered orally every 3 hours controlled the blood pressure at an average level of 120/70 mm. Hg. During phentolamine therapy, bioassay revealed less than 0.1 γ of norepinephrine per ml. of urine. On the twelfth day of hospitalization a tumor weighing 66 grams was removed from the chest. Manipulation prior to excision produced an increase in blood pressure from 90/60 mm. Hg to 190/120 mm. Hg. The tumor contained areas histologically characteristic of both pheochromocytoma and ganglioblastoma. After the surgical procedure the blood pressure stabilized at 120/70 mm. Hg without further treatment. No further sweating or tachycardia was observed. A fourth bioassay for catechol amines revealed less than 0.1 γ per milliliter of urine. Two years after leaving the hospital the patient was normotensive and feeling well.

Case Report 2.—A 20-year-old white man entered Barnes Hospital 3 weeks after the sudden onset of weakness and lightheadedness. On admission his pulse was 100 beats per minute, his blood pressure was 270/170 mm. Hg, but his temperature was normal. Pertinent physical findings included hemorrhagic and exudative retinitis with papilledema and left costovertebral angle tenderness with no palpable abdominal masses. Despite marked proteinuria and microscopic hematuria, renal function was excellent. The fasting glucose level was 74 mg. per 100 ml. of blood. The basal metabolic rate was 34 and 40 per cent above normal. Intravenous and retrograde pyelography, as well as roentgenograms after the injection of presacral air, were normal. A phentolamine test performed immediately after sedation was discontinued produced a fall in blood pressure of 50/35 mm. Hg, but a second test performed later had no effect. Bioassay revealed the pressor equivalent of 0.4 γ of norepinephrine per milliliter of urine. In preparation for surgical procedure 100 mg. of phentolamine was injected intravenously over a period of a few minutes, lowering the blood pressure from 270/170 to 240/145 mm. Hg. The addition of 100 mg. of hydralazine (Apresoline) orally further lowered the diastolic pressure to 120 mm. Hg at which time exploration was performed. Manipulation of a left adrenal tumor caused the blood pressure to rise markedly. After excision of the mass, the blood pressure became unobtainable and norepinephrine was started intravenously. Within 12 hours the blood pressure stabilized at 130/90 mm. Hg without drugs. After 4 days the papilledema was noted to be disappearing rapidly. Seven days postoperatively

bioassay for catechol amines revealed less than 0.1 γ norepinephrine per milliliter of urine. One year after the operation his blood pressure and ocular fundi were normal and he was feeling well.

RESULTS

Pharmacologic Results.—The addition of 2 γ norepinephrine to each of the 20 ml. baths surrounding strips from the aortas of 100 different rabbits produced an average shortening of 30.9 per cent with a standard deviation of 1.4 per cent. Quadrupling and successively quartering the concentration of norepinephrine gave the results shown in Table I. The contraction produced by epinephrine

TABLE I. PHARMACOLOGIC RESULTS

NO. OF ASSAYS	PRESSOR COMPOUNDS	CONCENTRATION (γ/L.)	SHORTENING (%)	ACTIVATOR OR INHIBITOR	CONCENTRATION (γ/L.)	FURTHER CHANGE (%)
100	Norepinephrine	100	30.9			
4	Norepinephrine	1.6	3.1			
4	Norepinephrine	6.3	14			
4	Norepinephrine	25	23			
4	Norepinephrine	100	30			
4	Norepinephrine	400	34			
4	Epinephrine	100	25			
4	Tryptamine	600	15			
4	Tyramine	1,750	7*			
4	Isoamylamine	5,000	3*			
4	Serotonin	250	13			
4	Angiotensin	20	12			
4	Norepinephrine	100	30	Cocaine	1,000	+3†
4	Norepinephrine	100	30	Dibenamine	100	-30‡
4	Norepinephrine	100	32	Phentolamine	100	-32‡
4	Norepinephrine	100	29	Hydralazine	1,000	0
4	Norepinephrine	100	29	Reserpine	25	0
4	Norepinephrine	100	29	Chlorothiazide	50,000	0
4	Norepinephrine	100	31	Pentolinium	50,000	0
4	Norepinephrine	100	31	Mecamylamine	50,000	0
4	Norepinephrine	100	31	Phenobarbital	50,000	0
4	Norepinephrine	100	31	Morphine	50,000	0
4	Tryptamine	600	15	Cocaine	1,000	0
4	Angiotensin	1.0	16	Dibenamine	100	0

*Ten thousand γ gave no further contraction.
†Further contraction.
‡Complete relaxation.

and by some other pressor agents is tabulated, as is the enhancement of norepinephrine by cocaine and its inhibition by Dibenamine. Also indicated are the maximum concentrations of antihypertensive agents like hydralazine, reserpine, chlorothiazide, and ganglion blockers as well as barbiturates and narcotics which produced neither inhibition nor enhancement of the effect of added norepinephrine.

Clinical Results.—Of the 147 patients, 9 had the pressor equivalent of 4 to 0.4 γ of norepinephrine per milliliter of urine. The remaining 138 had the equivalent of less than 0.1 γ per milliliter of urine. Some pertinent clinical information about the 9 patients with high excretions of catechol amine is

summarized in the first part of Table II. All of these patients were hypertensive and in all but the second, third, and seventh the hypertension was considered to be paroxysmal. Eight of the 9 had phentolamine tests, all of which were characterized by decreases in blood pressure ranging from 45/35 to 145/60 mm. Hg. The same 8 were explored surgically and 6 were found to have pheochromocytoma, 4 on the right and 2 on the left. For each of these 6, several urinary assays were performed. Three consistently had more than 0.3 γ norepinephrine per milliliter of urine. The 30-month-old girl and the 20-year-old man apparently had less than 0.3 γ norepinephrine per milliliter of urine when they were receiving daily oral doses of 80 and 2,400 mg. of regitine, respectively; while without such medication they excreted the larger amounts indicated in the table. Similarly the 27-year-old man had less than 0.3 γ of norepinephrine per milliliter of urine during the 8 hours following an intramuscular injection of 65 mg. of morphine sulfate; later he also excreted the larger amounts indicated in the table.

Three of the 9 patients who excreted the equivalent of more than 0.3 γ of norepinephrine per milliliter of urine did not have proved pheochromocytoma. The 61-year-old man had an adrenal tumor which was difficult to classify pathologically but which resembled the tumor of primary aldosteronism. His hypertension has apparently persisted after the excision of the tumor, although his case has not been carefully followed. The 34-year-old woman was extremely obese, being 165 cm. tall and weighing 130 kilograms. She excreted from 0.4 to 0.6 γ of norepinephrine per milliliter of urine on 3 occasions. At subsequent exploratory laparotomy, careful palpation of the adrenals revealed marked hypertrophy bilaterally without evident tumor; adrenal manipulation produced no rise in blood pressure. After removal of an acutely inflamed gall bladder and subtotal adrenalectomy, 2 assays revealed less than 0.1 γ of norepinephrine per milliliter of urine. The 20-year-old woman immediately left the hospital when confronted with the prospect of an operative procedure.

The second part of Table II summarizes the clinical data for the 138 patients with normal excretions of catechol amine. All of these patients had some elevation of blood pressure. On the average their hypertension had been recognized for 8.6 years; in 69 it was considered paroxysmal. Of the 61 patients who had phentolamine tests, 10 had decreases in blood pressure of more than 35/25 mm. Hg; the maximum change was 45/35 mm. Hg. Four of the 10 patients were sedated at the time of testing and later had insignificant decreases in pressure after sedation had been discontinued.

DISCUSSION

Provocative tests for the diagnosis of pheochromocytoma are potentially dangerous, and even injection of phentolamine is contraindicated in the presence of angina, arrhythmia, or threatened thrombotic stroke. On the other hand, there are no contraindications to assaying urinary catechol amines. In patients receiving sedatives, narcotics, or antihypertensive drugs, phentolamine has been reported to produce both marked decreases of blood pressure in the absence of pheochromocytoma and minimal changes in its presence.[22, 23] Uremia or severe

TABLE II. CLINICAL RESULTS

PATIENT			HISTORY			PHYSICAL			URINE			BLOOD			OTHER TESTS			SPECIFIC TESTS			SURGERY	
AGE (YEARS)	RACE	SEX	DURATION OF HYPERTENSION (YRS.)	SWEATS	PALPITATION	PULSE	SYSTOLIC PRESSURE	DIASTOLIC PRESSURE	PROTEIN	GLUCOSE	PSP (% EXCRETED IN 15 MIN.)	NONPROTEIN NITROGEN (MG. %)	FASTING GLUCOSE (MG. %)	PBI (γ%) OR BMR (%)	ECG ABNORMALITY	CARDIOMEGALY ON X-RAY	ADRENAL ABNORMALITY ON X-RAY	HISTAMINE TEST	PHENTOLAMINE TEST	CATECHOL AMINE ASSAY (γ/ML.)	TISSUE EXCISED	
Part I: Individual Clinical Data for 9 Patients With Elevated Levels of Urinary Catechol Amine.—																						
2.5	N	F	0.8	+	+	140	190	120	+	0							Chest		+	0.8	Pheochromocytoma	
20	W	M	0.1	+	+	100	250	175	+	0	60	27	74	+40%	0	+	+		+	0.4	Pheochromocytoma	
61	W	M	8	+	+	88	280	180	+	+		86	152		PVC	0	+		+	0.5	Pheochromocytoma	
35	W	F	8	+	+	92	180	120	+	0	30	17	130	4.2γ%	0	0	+		+	4.0	Pheochromocytoma	
62	W	M	10	0	+	62	225	100	0	0	25	19	170	+10%	AVC	+	+		+	0.6	Pheochromocytoma	
27	W	M	8	+	+	120	290	160	0	0	35	31	78	+11%	LVE	+	0		+	0.7	Pheochromocytoma	
61	W	M	12	+	+	100	280	140	+	0	30	26	93	>20γ%	LVE	+	+		+	0.6	Adrenal tumor	
34	W	F	10	+	+	104	220	115	0	0	30	21	77	−15%	0	+	+		+	0.5	Large adrenals	
20	W	F	12	+	+	100	145	100	0	0		12		5.2γ%						0.5	None	
Part II: Summary of Clinical Data for 138 Patients With Normal Levels of Urinary Catechol Amines.—																						
% Abnormal			3%	6%	14%	89%	77%	49%	4%	52%		22%	17%	7%	73%	42%	2%	7%	14%	0%		0%

In Part I the column designated PSP records the per cent of 6 mg. of intravenously injected phenol red which appeared in the urine of adequately hydrated patients within 15 minutes. Because zinc hydroxide was used to precipitate blood proteins, the maximum normal levels of circulating nonprotein nitrogen and fasting glucose were 25 and 90 mg. per 100 ml. of plasma, respectively. Both protein-bound iodine and basal metabolic rate are tabulated in the same column, the appropriate units being indicated. The abbreviations for the electrocardiographic abnormalities are as follows: PVC for premature ventricular contractions, AVC for abnormal ventricular conduction, and LVE for left ventricular enlargement. The criterion for a positive histamine test was a greater increase in blood pressure following the subcutaneous injection of 0.2 mg. of histamine than after the immersion of one hand in ice water for 2 minutes. The criterion for a positive phentolamine test was a fall in blood pressure of more than 35/25 mm. Hg which persisted for at least 2 minutes. Catechol amine levels are expressed in terms of their norepinephrine equivalent.

In Part II the figures indicate the percentage of patients examined who had *abnormal* findings. The age, race, sex, and duration of hypertension are omitted since they are given in the text. The maximum normal pulse was considered to be 100 beats per minute and the maximum normal blood pressure 150/100 mm. Hg. Data were available for all 138 patients except in the columns headed PSP, Fasting Glucose, PBI or BMR, Adrenal Abnormal on X-Ray, Histamine Test, Phentolamine Test, and Tissue Excised, in these columns the numbers of patients involved were 70, 122, 67, 74, 14, 69, and 11 patients, respectively. Surgical exploration of the 11 patients revealed that 5 had hypoplastic kidneys, 2 had renal neoplasms, 2 had renal cysts, and 2 had normal kidneys and adrenal glands.

emotional disturbance may also be associated with misleading phentolamine tests.[8, 22, 24] Our data do not indicate that azotemia, barbiturates, or the usual antihypertensive agents have any effect on the bioassay procedure described. On the other hand in patients with proved pheochromocytoma the apparent excretion of catechol amine was low after parenteral morphine or therapeutic doses of oral phentolamine, and excretion rose when administration of these drugs was discontinued.

SUMMARY

A bioassay technique for the estimation of the level of catechol amine in urine with the use of spirally cut strips of rabbit aorta is considered. The activities of physiologic concentrations of catechol and other amines in the system are tabulated in order to facilitate interpretation of the effects of human urine. Pharmacologic data are given for compounds which differentially potentiate and inhibit such effects. Some clinical data have been collected for 147 hypertensive patients on whom bioassays of urinary catechol amines were done because of suspected pheochromocytoma. Of these patients, 6 with surgically proved pheochromocytoma and 3 for whom that pathologic diagnosis could not be made, excreted the pressor equivalent of more than 0.3 γ of norepinephrine per milliliter of urine; 8 who were tested had marked decreases in blood pressure after administration of phentolamine. None of the other 138 excreted as much as 0.1 γ of norepinephrine per milliliter of urine, although 10 per cent of those tested had marked responses to phentolamine.

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INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY HEMOLYSIS INDUCING DRUGS

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IT HAS been found that patients suffering from hemolytic anemia caused by certain drugs are deficient in erythrocyte glucose-6-phosphate dehydrogenase (G-6-P D)[1-4] and have abnormal diminution of reduced glutathione in the red cell upon in vitro exposure to acetylphenylhydrazine.[5, 6] This investigation was carried on to study the possibility of interaction between these drugs and glucose-6-phosphate hydrogenase.

Enzyme inhibitors may be classified as competitive or noncompetitive. In competitive inhibition, the inhibitor, which is usually similar in structure to the substrate, may either compete with the substrate in combining with the active portion of the enzyme and prevent the formation of the enzyme-substrate complex or may compete with the coenzyme for the apoenzyme. In noncompetitive inhibition, the activity of the coenzyme or the apoenzyme is decreased in proportion to the concentration of the inhibitor but is independent of substrate or coenzyme concentration. The activity of the coenzyme may be reduced by the combination with such radicals as H_2S, HCN, or NH_2, or by binding of free ions which may be necessary for the enzyme activity. Inhibition of the apoenzyme may occur when the catalytic effect of functional groups upon whose activity the enzyme is dependent is destroyed or reduced by their combination with other substances. Further metabolism of the product of the enzymatic reaction may also be inhibited.

The present experiments were designed to investigate the possibility of direct inhibition of G-6-P D by the drugs which have been reported as hemolytic agents in drug sensitive individuals.

METHODS AND MATERIALS

Buffer.—Tris (hydroxymethyl) aminomethane buffer was dissolved in acidified distilled water to prepare a 0.035M solution, final pH of 7.4.

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Substrates.—Disodium glucose-6-phosphate (G-6-P) and sodium 6-phosphogluconate (6-P G) were obtained from Sigma Chemical Co. Solutions of glucose-6-phosphate varying in concentration from 1.6×10^{-4} to 8.2×10^{-3} moles per liter and 6-phosphogluconate from 3.1×10^{-4} to 1.6×10^{-2} moles per liter were prepared by dissolving the salts in buffer.

Enzymes.—The commercially prepared enzymes, G-6-P D and 6-phosphogluconic dehydrogenase (6-P G D), prepared from ammonium sulfate fractions of yeast autolysate, were obtained from Sigma Chemical Co. Solutions containing 0.05 mg. per milliliter of G-6-P D and 2 mg. per milliliter of 6-P G D were prepared by dissolving the enzymes in buffer. The activity of both enzymes in solution gradually decreased. Therefore, fresh preparations were made each day and a control was run with each experiment. These concentrations allowed maximal activity of the enzyme with the highest concentration of substrate.

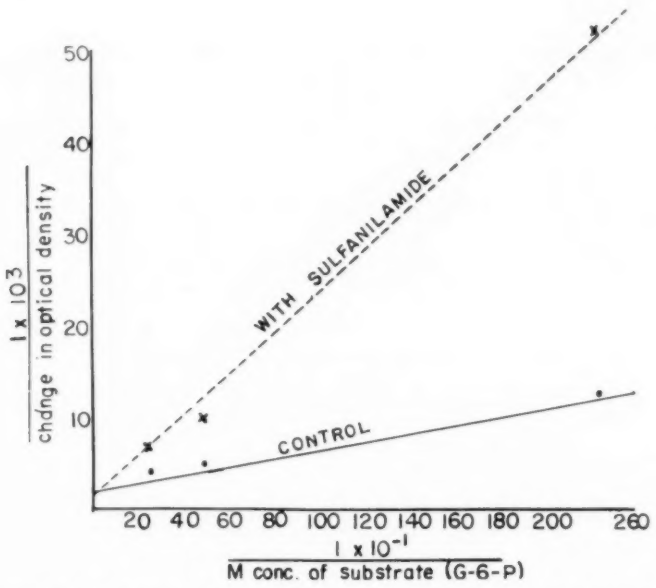

Fig. 1.—The effect of sulfanilamide on G-6-P activity in the presence of varying concentrations of substrate is plotted on the abscissa and the reciprocal of enzyme activity expressed as increment optical density units times 10^3 is plotted on the ordinate. The common intercept of the curves with and without inhibitor demonstrates competitive inhibition.

Hemolyzates.—The hemolyzates were prepared from packed red cells which had been washed with saline. A given volume of cells was diluted with an equal volume of distilled water, and a final dilution in water was made to 0.5 to 1.0 Gm. per cent hemoglobin concentration. This preparation was used within hours or was frozen at −25° C. until used. In experiments where hemolyzates were dialyzed, this was carried out at 8° C. for 16 hours in a volume of distilled water 150 times that of the hemolyzate.

Coenzymes.—Triphosphopyridine nucleotide (TPN) and reduced triphosphopyridine nucleotide (TPNH), prepared from yeast diphosphopyridine nucleotide, were obtained from Sigma Chemical Co. Solutions containing 0.01, 0.005, and 0.0025 moles of TPN per liter were prepared in 0.035M Tris buffer.

Inhibitors.—With the exception of B-naphthol, the materials to be tested were dissolved in 0.035M Tris buffer in the following concentrations: 3.5×10^{-5}M menadione sodium bisulfite (Hykinone), 0.001M solutions of p-chloromercuribenzoic acid, sulfadiazine, primaquine, acetyl phenylhydrazine, p-aminobenzoic acid, and tolbutamide (Orinase*). Because of its insolubility, 1 mg. of B-naphthol was placed in a test tube with an appropriate amount of buffer.

The experimental procedure was carried out by determining optimal conditions for inhibition and then observing effects of variation of concentration of substrate or coenzyme. Six tenths milliliter of the afore-mentioned solutions of inhibitor or 1 mg. of B-naphthol in 0.6 ml. of buffer was incubated with 0.8 ml. of the yeast enzyme. In experiments with the erythrocyte enzyme system, 0.3 ml. of hemolyzate was used instead of the yeast preparation. All were incubated for 30 minutes except menadione sodium bisulfite which required 2 hours for maximum inhibition in this system. Then 1.0 ml. aliquots of substrate of varying molarity as described, 0.4 ml. of 0.01M TPN, and 0.2 ml. of buffer were added to reach a final volume of 3.0 ml. Observations were carried out at 25° C. spectrophotometrically in a Beckman DU spectrophotometer by noting the increase in density at 340 mμ produced on the reduction of TPN. Readings were made every 5 minutes for 15 minutes, the increments of the optical density for these three 5 minute intervals were recorded and the average was calculated. The reciprocal of this average value, which corresponds to the observed velocity of the enzyme reaction, was then plotted against the reciprocal of the substrate or coenzyme concentration. A control curve was determined each time. By this technique, competitive inhibition is demonstrated by a greater slope of the inhibitor line than of the control line and common intercept of the 2 lines whereas noncompetitive inhibition is manifested by divergent intercepts.

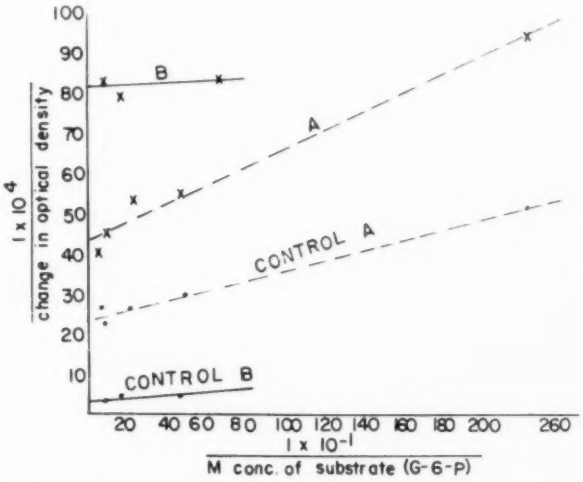

Fig. 2.—The effects of menadione sodium bisulfite *(A)* and acetylphenylhydrazine *(B)* on G-6-P D in the presence of varying amounts of enzyme. Ordinate and abscissa are plotted as in Fig. 1. The lack of common intercepts is consistent with noncompetitive inhibition. The effect of acetylphenylhydrazine is greater than that of menadione sodium bisulfite.

RESULTS

Observations of the effect of the inhibitors on yeast G-6-P D revealed 2 types of inhibition. In the presence of sulfanilamide, there was insignificant difference in the intercepts of the control and inhibitor curves but a significant difference in slope (Fig. 1). In contrast, primaquine, nitrofurantoin, tolbutamide, acetylphenylhydrazine, menadione sodium bisulfite, p-chloromercuribenzoic acid, and B-naphthol all manifested noncompetitive inhibition. The varying degree of inhibition is apparent in Fig. 2 in which results of 2 of these experiments have been plotted. The lack of competitive inhibition in these figures is demonstrated by the increased intercept with correspondingly less change

*Orinase was supplied by The Upjohn Company, Kalamazoo, Mich.

in slope. Acetylphenylhydrazine was the most effective inhibitor in the concentrations used, but repeated studies with this chemical revealed that the degree of inhibition was related to the age of the solution. Fresh preparations which were colorless caused little or no inhibition. Preparations which had been standing turned yellow to yellowish brown and showed marked inhibition. The color change was probably due to oxidation of the compound with the formation of a nitro group.

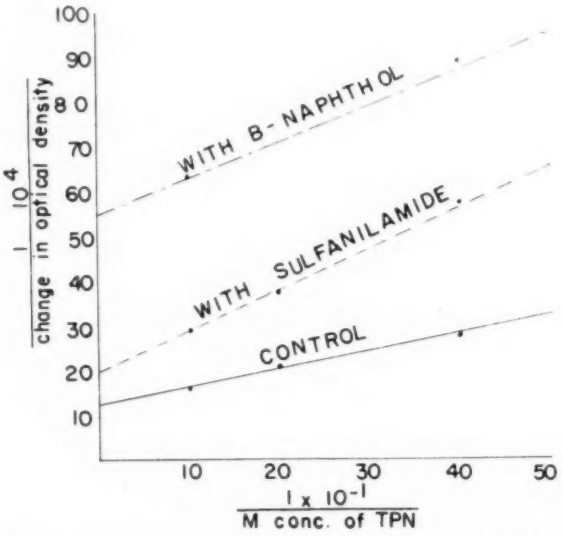

Fig. 3.—The effects of sulfanilamide and B-naphthol on G-6-P D in the presence of varying amounts of TPN. The reciprocal of the concentration of TPN is plotted on the abscissa and the reciprocal of enzyme activity on the ordinate. The curves demonstrate noncompetitive inhibition.

To determine if the drugs compete with the coenzyme as well as with the substrate, the concentration of TPN was varied, and the concentrations of the substrate, the enzyme, and the inhibitor were kept constant. Observations of the rates of reaction demonstrated no competition with the coenzyme. The results in the presence of sulfanilamide and B-naphthol are plotted in Fig. 3.

To determine if the drugs were acting as hydrogen acceptors for TPNH in the system, thereby interfering with accurate assay of its production, this effect on TPNH was evaluated. Primaquine, nitrofurantoin, sulfanilamide, and p-chloromercuribenzoate were incubated with 0.01M TPNH in the concentrations used in the inhibition experiments. Observations of O.D. at 340 mμ revealed no evidence of TPNH oxidation.

The effect of the drugs on 6-P G D was also determined to compare this action on another TPN dependent enzyme, closely related in the hexose monophosphate shunt. Fig. 4 indicates that sulfanilamide, menadione sodium bisulfite, and acetylphenylhydrazine are competitive inhibitors of 6-P G in that each inhibitor curve has a common intercept with the control, but a diverging slope. In contrast, primaquine (Fig. 5) is a noncompetitive inhibitor as i

nitrofurantoin, p-chloromercuribenzoate, B-naphthol, and tolbutamide. Thus, the type of inhibition of these 2 TPN dependent dehydrogenases by these drugs differs. Menadione sodium bisulfite and acetyl phenylhydrazine inhibit G-6-P D noncompetitively, whereas they act as a competitive inhibitor of 6-P G D. Table I contains a summary of the types of inhibition by these drugs.

The effect of several other drugs in these systems was also observed. Glutethimide, a drug chemically unrelated to any of those studied above and clinically not observed to cause hemolytic anemia, was found to have no inhibitory activity in concentrations of 0.0113M under the usual conditions of the test. Para-aminobenzoic acid was studied because of its known competitive action with sulfanilamide and because of its demonstrated lack of clinical effect in sensitive patients.[5] This also was ineffective as an inhibitor in the test system.

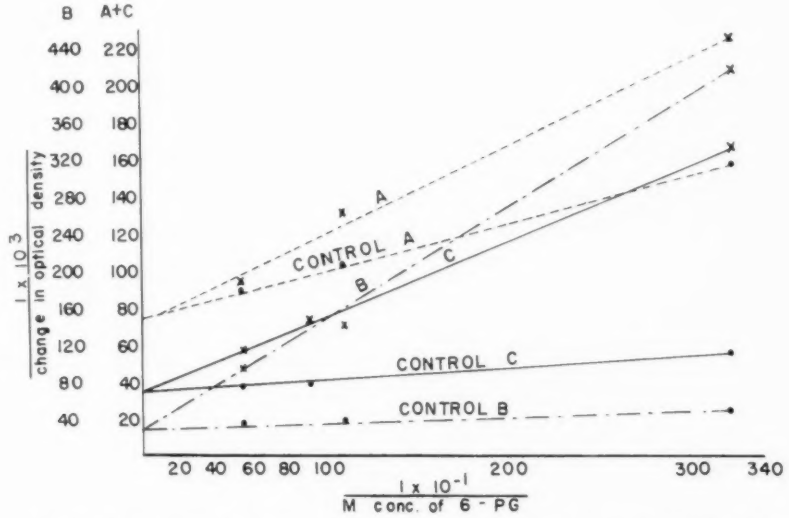

Fig. 4.—The effects of sulfanilamide (*A*), menadione sodium bisulfite (*B*), and acetylphenylhydrazine (*C*) on 6-P G D in the presence of varying amounts of substrate. Ordinate and abscissa are plotted as in Fig. 1. All 3 show competitive inhibition.

The Michaelis constants of G-6-P D and 6-P G D with respect to substrate and coenzyme have been calculated (Table II). G-6-P D has a greater affinity both for TPN and for substrates than does 6-P G D.

TABLE I. SUMMARY OF MECHANISMS OF INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND OF 6-PHOSPHOGLUCONATE DEHYDROGENASE BY DRUGS

INHIBITOR	GLUCOSE-6-PHOSPHATE DEHYDROGENASE	6-PHOSPHOGLUCONATE DEHYDROGENASE
Menadione sodium bisulfite	Noncompetitive	Competitive
Nitrofurantoin	Noncompetitive	Noncompetitive
Sulfanilamide	Competitive	Competitive
Primaquine	Noncompetitive	Noncompetitive
P-chloromercuribenzoate	Noncompetitive	Noncompetitive
B-naphthol	Noncompetitive	Noncompetitive
Acetylphenylhydrazine	Noncompetitive	Competitive
Tolbutamide	Noncompetitive	Noncompetitive

Observations of the hemolyzate preparations revealed similar inhibition of both enzyme activities. Noncompetitive inhibition by the same drugs was observed in varying degree. The results of an experiment with one of these, primaquine, has been plotted in Fig. 6. Sulfadiazine was again noted to be a competitive inhibitor of G-6-P D activity. The kinetics of inhibition by this

TABLE II. MICHAELIS-MENTEN CONSTANTS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE WITH SUBSTRATE AND WITH TRIPHOSPHOPYRIDINE NUCLEOTIDE

ENZYME	K_S	K_{TPN}
Glucose-6-phosphate dehydrogenase	4.7×10^{-9}	5×10^{-9}
6-phosphogluconate dehydrogenase	1.89×10^{-8}	1.6×10^{-7}

drug may be compared to those of primaquine in Fig. 6. The results of investigations into the type of inhibition of activity of G-6-P D and 6-P G D activity in the hemolyzates must, however, be interpreted with some caution since other enzymes of the glycolytic cycle may use the substrate or the products of the reaction.

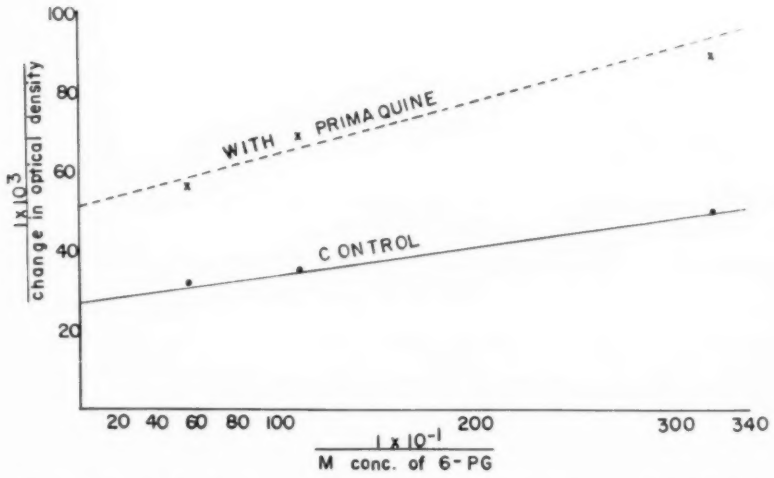

Fig. 5.—The effect of primaquine on 6-P G D in the presence of varying concentrations of substrate. Ordinate and abscissa are plotted as in Fig. 1. Primaquine is a noncompetitive inhibitor.

Acetylphenylhydrazine had a variable effect on hemolyzates depending upon the age of the solution. Preparations which had been standing long enough to become yellow caused inhibition similar to that seen with the purified yeast preparation. Freshly prepared solutions, however, not only showed no inhibition of G-6-P D but actually resulted in an increase in O.D. at 340 mμ on incubation with hemolyzates. When TPN and the drug were incubated without hemolyzate no significant change occurred. To determine whether the freshly prepared solutions had a reverse effect and actually did lead to increased production of TPNH, experiments were carried out in the absence of TPN. When

the drug was incubated with the hemolyzate after the hemolyzate had been dialyzed overnight against distilled water at 8° C., there was an increment in O.D. reading during the period of observation. Absence of TPN in the dialyzed hemolyzate could be demonstrated by a lack of TPNH production when G-6-P was added to the hemolyzate. Upon incubating acetylphenylhydrazine with both hemolyzate and TPN a greater change occurred (Fig. 7). Since many substances absorb light at 340 mμ, one cannot state the compound formed. However, it is evident that under the conditions of the experiment inhibition of G-6-P D by acetyl phenylhydrazine can be demonstrated only after the solution was aged.

Thus, these agents act as inhibitors of both G-6-P D and 6-P G D in either competitive or noncompetitive fashion. Such action may be demonstrated in crude human hemolyzates as well as isolated enzyme preparations from yeast.

DISCUSSION

There are few reports on studies of drug inhibition of G-6-P D. The reaction of antimalarial drugs, Atabrine and quinine, has been investigated by Haas.[9] In his study of the effects of therapeutic agents on the components of

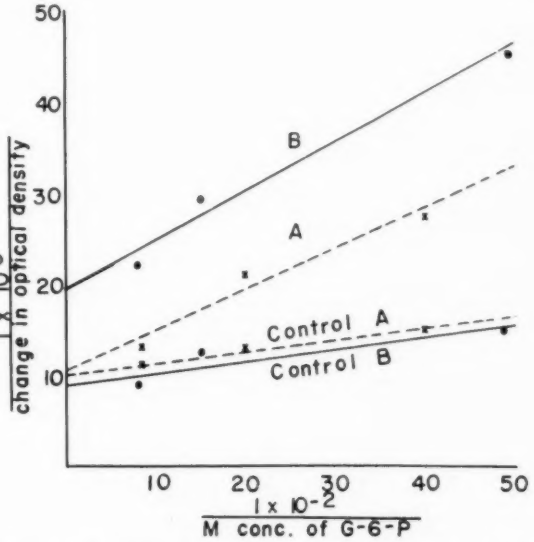

Fig. 6.—The effect of sulfanilamide *(A)* and primaquine *(B)* on G-6-P D activity in a hemolyzate preparation. Ordinate and abscissa are plotted as in Fig. 1. Sulfanilamide shows competitive and primaquine noncompetitive inhibition.

respiratory enzyme system, he demonstrated inhibition of the enzyme by Atabrine and postulated displacement both of the coenzyme and the substrate by the drug. Quinine was found to be much less effective than Atabrine. Alt-[10] in his work on the effect of sulfonamides on respiratory enzymes, found that G-6-P D is strongly inhibited by sulfonamides, the most effective of which was found to be sulfanilamide. He, too, suggested that the drugs may compete

with both the substrate and coenzyme. Recently renewed interest in drug action on this enzyme system has been aroused by the demonstration by Alving and others[1, 2] that certain types of drug-induced hemolysis are related to deficiency of erythrocyte G-6-P D. Beutler[11] has suggested that these drugs act in the red cell by modifying hemoglobin, with this in turn leading to oxidation of glutathione. The demonstration by Mills of the presence of glutathione peroxidase in the red cell suggests that reduced glutathione may act as hydrogen donor in the presence of oxidant drugs, thus sparing hemoglobin.[12] An enzymatic defect of G-6-P D would then be evident by the lack of reduction of this oxidized glutathione by glutathione reductase which requires the generation of TPNH from the G-6-P D and 6-P G D reactions. Thus, the drugs would exaggerate a defect by placing an increased demand on a limited system.

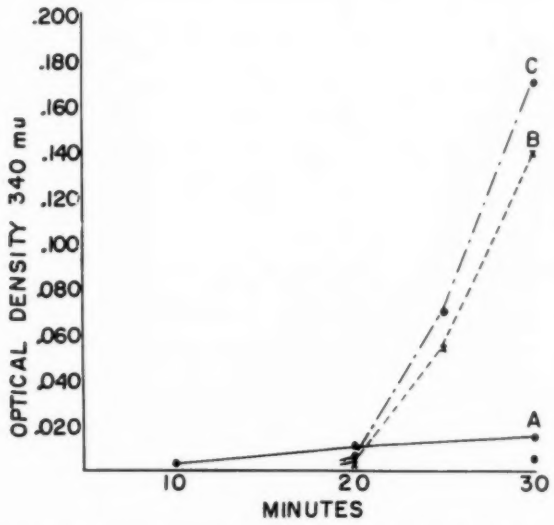

Fig. 7.—Change in optical density at 340 mμ produced by incubation of acetylphenylhydrazine with a hemolyzate. Optical density is plotted on the abscissa and time of incubation is plotted on the ordinate. Results of incubation of the drug with TPN alone are shown on curve *A*, of incubation with hemolyzate are shown on curve *B*, and of incubation with both hemolyzate and TPN on curve *C*.

Although the present studies do not negate a remote effect of drugs such as that postulated, they clearly demonstrate that there is a significant direct inhibitory action of these drugs on yeast G-6-P D and 6-P G D as well as on the crude preparations of these enzymes in erythrocytes. The mechanisms of action of the different drugs which cause hemolytic anemia on the erythrocyte in vitro are not the same since they vary both in degree and type of inhibition. This observation is reasonable since the drugs studied have different components, structures, and properties.

That an inhibitor is competitive is determined by the sites involved in the reaction. A chemical reaction between sulfanilamide and TPN occurring at the oxidation-reduction site in the coenzyme molecule with the formation of a new compound could explain the mechanism of the inhibition caused by sulfanil-

amide and related drugs toward TPN linked dehydrogenases. Here, the inhibitor competes with the substrate for the active site, which is the para position of the pyridine ring, in the coenzyme molecule. The theory is consistent with the results presented as well as with Altman's although the fact that he found competition of the drug with the coenzyme remains unexplained. In our studies, competition with coenzyme for apoenzyme could not be demonstrated.

Competitive inhibition of drug with substrate in these reactions may depend on the affinity of the substrate for the coenzyme. Calculation of the Michaelis constants of the dehydrogenases has demonstrated that TPN is more firmly bound to G-6-P D than to 6-P G D which confirms the findings of Glock and McLean[13] concerning the affinity of the enzyme to the coenzyme. This fact may play some part in the kinetics of the enzyme inhibition and explain the observation that menadione sodium bisulfite and acetylphenylhydrazine are effective competitive inhibitors for substrate in the 6-P G D but not in the G-6-P D reaction.

Noncompetitive inhibition of both enzymes by primaquine, nitrofurantoin, B-naphthol, and p-chloromercuribenzoate probably reflects similar action of certain groups of the coenzyme or apoenzyme. According to Dubnoff and Barton[14] the activity of G-6-P D is dependent upon the presence of SH groups. The noncompetitive inhibition may, therefore, be explained by the inactivation of these since most of these compounds are known to be potent inhibitors of sulfhydryl enzymes. In the case of B-naphthol, the phenol is oxidized first to a quinone which then combines with the SH group, thus inhibiting the action of the enzyme. Nitrofurantoins are known to act as electron acceptors for certain respiratory enzymes,[15] and it is possible that nitrofurantoin may interfere with electron transfer in these dehydrogenase systems.

Thus, both TPN dependent dehydrogenases, which are the only known source of TPNH in the erythrocyte, are inhibited by drugs which have been reported to cause hemolytic anemia in sensitive patients as well as by p-chloromercuribenzoic acid, a known SH inhibitor. That inhibition of G-6-P D has been described with Atabrine and quinine,[9] which have not been reported to be hemolytic agents, suggests that other factors are important in the hemolytic action of these drugs. It should also be emphasized that the relative concentration of drug to enzyme is greater in these in vitro studies than would occur in vivo. However, the experiments were designed for maximal effects in short-term studies and could certainly be reflecting an effect which is less marked but more prolonged in vivo. These actions in the presence of deficient G-6-P D could play some part in hemolysis by interfering with adequate production of TPNH. The importance of TPNH in metabolic processes such as glutathione and methemoglobin reduction as well as in lipogenesis is well recognized but it is not certain that these are crucial for the survival of the adult red cell. The primaquine sensitive erythrocyte has multiple enzymatic abnormalities[16, 17] and it is not yet known which, if any, of these is responsible for the drug-induced hemolysis. That the drugs inhibit G-6-P D in an in vitro system must then be regarded as evidence for only one of many possible mechanisms in the pathogenesis of this disease.

SUMMARY

Certain drugs which are known to cause hemolytic anemia in susceptible individuals have been shown to inhibit glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase derived from yeast or crudely prepared from erythrocytes as an hemolyzate. Both competitive and noncompetitive type inhibition of both enzymes was observed.

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PLASMA ELECTROLYTE COMPOSITION IN DOGS BREATHING HIGH CO_2 MIXTURES: SOURCE OF BICARBONATE DEFICIT IN SEVERE RESPIRATORY ACIDOSIS

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IN THE early days of our studies with high carbon dioxide concentrations in dogs, it was observed that with prolonged breathing of mixtures with a high percentage of CO_2 metabolic acidosis regularly appeared superimposed on the severe respiratory acidosis which was being induced.[1] Other investigators have reported similar observations.[2] This study is an attempt to determine the source of this bicarbonate deficit by a complete electrolyte analysis of plasma from dogs before, during, and after they breathe mixtures with a high percentage of CO_2.

METHODS

The procedure used in this laboratory for exposing dogs to 2 hours' breathing of 30 per cent CO_2 followed by 2 hours' breathing of 40 per cent CO_2 has been described previously.[3] A control 30 ml. sample of blood was drawn from a femoral artery after the animal was anesthetized with intravenous thiopental sodium. Forty milliliter samples were drawn at the end of the 4 hour period of breathing mixtures with a high percentage of CO_2, and 15 minutes after return to air breathing. All blood samples were handled anaerobically. Immediately after the blood was drawn, the cells were spun down and the plasma was taken up into sealed syringes. The pH was determined on a sample of whole blood at 37.5° C. by means of a glass electrode pH meter. Total CO_2 content of plasma was determined by the manometric Van Slyke method and bicarbonate concentration and P_{CO_2} were calculated from these 2 measurements by the Henderson-Hasselbalch equation using 6.11 for pK′. Chloride was measured by a modified Volhard method,[4] phosphate by the Fiske-Subbarow technique,[5] protein by the micro-Kjeldahl method, sodium and potassium by flame photometry on a protein-free filtrate, and calcium and magnesium by a combined technique described by Sobel and Hanok.[6] R is the difference between total cations and total anions. The bicarbonate concentration which should be present at pH 7.4 was calculated as follows:

$$(HCO_3^-)_{7.4} = (HCO_3^-)p - [\,(7.4 - pH)\,(8.2 + 2.3\ Hgb.)\,] \qquad (1)$$

in which (8.2 + 2.3 Hgb.) is the slope of the CO_2 absorption curve plotting $(HCO_3^-)p$ against pH. Hgb. is hemoglobin concentration expressed as millimoles per liter. Phosphorus concentration was converted from milligrams per cent to milliequivalents per liter using 6.8 as pK. The milliequivalent value for protein was calculated from grams per cent by the equation given by Peters and Van Slyke[7] assuming an albumin:globulin ratio of 1.8. This equation is expressed as follows:

$$\text{milliequivalents per liter protein} = 0.125\ (\text{albumin})\ (pH - 5.16) + 0.077\ (\text{globulin})\ (pH - 4.89) \qquad (2)$$

Albumin and globulin are expressed as grams per liter.

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TABLE I. MEAN VALUES FOR 7 EXPERIMENTS

PLASMA	MEAN, STANDARD ERROR, AND P	PLASMA IONS (MEQ./L.)								TOTAL MEASURED ANIONS	TOTAL CATIONS
		HCO_3	CL	P	PR	R	NA	K	CA + MG		
Control	Mean	23.1	114.9	2.98	15.3	3.7	149.9	3.9	6.1	156.4	160.1
	S. E.	1.11	1.95	0.53	0.53	1.27	2.14	0.14	0.21	1.36	2.32
4 hrs. on CO_2	Mean	32.6	110.2	5.83	11.8	4.8	152.1	6.4	6.8	160.5	165.2
	S. E.	0.89	1.52	0.61	0.66	1.18	2.08	0.39	0.54	1.23	2.19
	p	<0.01	<0.01	<0.01	<0.01			<0.01		<0.01	<0.01
15 min. off CO_2	Mean	22.9	112.2	5.91	15.2	4.1	147.9	6.3	6.1	156.3	160.5
	S. E.	1.13	1.69	0.73	0.77	1.07	2.60	0.47	0.45	1.54	2.52
	p		0.05	<0.01			(<0.01)	<0.01			

These same determinations were made on 2 samples of dog blood equilibrated with 10 and 30 per cent CO_2, respectively.

RESULTS

Table I presents mean values with their standard errors for the various plasma ions in the 7 in vivo experiments. The p values given are for differences between the control sample and the sample indicated, except the value given in parentheses which is the p value for the difference between Samples 2 and 3. Table II is a similar table for total CO_2, pH, carbon dioxide tension, hemoglobin concentration, HCO_3^- at pH 7.4, and protein concentration. The usual decrease in pH from 7.37 to 6.71 with an increase in P_{CO_2} from 45 to 280 is apparent.

TABLE II. MEAN VALUES FROM 7 EXPERIMENTS

PLASMA	MEAN, STANDARD ERROR, AND P	TOTAL CO_2 (MM./L.)	pH	P_{CO_2} (MM. HG)	HGB. (MM./L.)	PROTEIN (GM. %)	(HCO_3^-) (PH 7.4)	
Control	Mean	24.4	7.37	45.1	7.8	6.3	21.6	
	S. E.	1.19	0.02	3.3	0.18	0.20	0.9	
4 hrs. on CO_2	Mean	41.0	6.71	279.0	9.9	6.8	10.9	14.5
	S. E.	1.45	0.05	25.5	0.20	0.30	1.7	1.6
	P				<0.01	<0.01		
15 min. off CO_2	Mean	24.4	7.28	51.9	9.8	6.4	19.3	
	S. E.	1.21	0.02	3.6	0.18	0.28	1.4	
	P				<0.01			

With this change in carbon dioxide tension, total CO_2 rose from 24.4 to 41 mM. per liter. A significant rise in hemoglobin from 7.8 to 9.9 mM. per liter took place during the breathing of mixtures with a high percentage of CO_2. This observation has been reported previously.[8] A significant rise in protein concentration is also apparent during the severe respiratory acidosis. This increase is probably due to a shift of water from plasma into intracellular spaces.[8] Fifteen minutes after CO_2 breathing was discontinued, total CO_2 had returned to its original value; however, pH was still low and P_{CO_2} was still high. This is evidence of a metabolic component in the acidosis which is present at this time, as can be seen by comparing the HCO_3^- concentration which would exist at pH 7.4 in the 2 cases. In 15 minutes the increase in hemoglobin concentration, which is due to contraction of the spleen, has not disappeared whereas the protein concentration has returned to control value.

Although the mean P_{CO_2} when the dogs were breathing 40 per cent carbon dioxide is approximately the value that might be expected, the very large standard error indicates that the mixture was not always exactly 40 per cent CO_2. With the increase in bicarbonate concentration from 23.1 to 32.6 mEq. per liter, chloride regularly decreased as required by the chloride shift. Inorganic phosphate concentration, expressed as milligrams per cent of phosphorus, regularly increased during acidosis; even when this value was calculated as milliequivalents per liter the change amounted to a twofold increase. The increase in protein concentration expressed as milliequivalents per liter is due

TABLE III. VALUES FROM THE EXPERIMENT WITH LARGEST HCO_3 DEFICIT

PLASMA	HGB.	HCO_3	HCO_3 at 7.4	$\triangle HCO_3$	CL	P	PR	R	NA	K	CA AND MG	TOTAL CATIONS	TOTAL ANIONS
Control	8.3	24.9	22.2	0.0	120.8	2.5	15.7	1.7	154.5	3.7	7.4	165.6	163.9
CO_2	9.2	34.4	11.5	10.7	112.4	7.6	10.4	2.1	150.0	7.2	9.7	166.9	164.8
After CO_2	9.4	20.7	15.9	6.3	116.5	7.9	14.4	3.8	148.0	6.8	8.5	163.3	159.5

TABLE IV. DATA FROM 2 IN VITRO EXPERIMENTS (mEq./L. PLASMA)

EXPERIMENT (% CO_2)	HGB. (MM./L.)	HCO_3	HCO_3 pH 7.4	CL	P	PR	R	NA	K	CA AND MG	TOTAL CATIONS	TOTAL ANIONS
1 (10)	10.2	21.8	18.6	115.4	2.4	16.1	5.7	151.5	3.6	6.3	161.3	155.6
1 (30)	10.2	35.4	16.4	108.1	1.9	13.1	11.4	160.0	3.6	6.3	169.9	158.5
2 (10)	6.9	18.6	13.8	118.0	3.8	13.0	−1.2	142.5	4.3	5.4	152.2	153.4
2 (30)	6.9	29.6	12.7	113.7	3.3	10.0	2.3	149.0	4.4	5.5	158.9	156.6

simply to the fact that the calculated binding power of protein decreases as pH approaches the isoelectric point (2). As seen previously, there was an actual increase in protein concentration in terms of grams per 100 ml. of plasma. The total of these 4 anions increased a significant amount during the 4 hours of respiratory acidosis. Both sodium and potassium showed an increase during the breathing of high per cent CO_2, although the increase in sodium was not statistically significant. The slight increase in calcium plus magnesium was also not a significant change. R represents unanalyzed anions and is simply the difference between total cation concentration and total measured anion concentration. The mean value for R found in these studied is somewhat low compared with the values given by others.[9] After 15 minutes of breathing air which followed the respiratory acidosis, bicarbonate concentration had returned to control value, chloride had increased, phosphorus had not changed, protein had returned to control value, sodium had decreased significantly below the value during acidosis, potassium had remained high, and total anions and total cations had returned to control value.

In order to determine the metabolic component which developes during the respiratory acidosis, bicarbonate concentrations which would exist at pH 7.4 were calculated (Table II). The value which bicarbonate concentration would have at pH 7.4 has been calculated in 2 ways for the blood sample at low pH: (1) the hemoglobin concentration that existed at the time blood Sample 2 was drawn was used and (2) the hemoglobin concentration that existed at the time Sample 1 was drawn was used. These 2 values represent the extremes that could be expected for bicarbonate concentration at pH 7.4 as a result of the change in hemoglobin concentration that occurred during the breathing of high percentage CO_2. The mean bicarbonate deficit which existed at the time Sample 2 was drawn was at least 7.1 and at most 10.7 mEq. per liter. However, after 15 minutes of breathing air this bicarbonate deficit had decreased to a very low value, that is, very little metabolic acidosis was present by 15 minutes after breathing CO_2 was terminated.

Experiment 7 showed the largest bicarbonate deficit on Sample 3, and the values for this experiment are presented in Table III. The bicarbonate deficit present in blood Sample 3 in this experiment appears to be due primarily to a decreased sodium concentration and an increased phosphorus concentration; thus, in spite of the fact that chloride was 4 mEq. below control value and potassium was 3 mEq. above control value, a deficit of bicarbonate still existed. It seems likely that these 2 factors also played a part in the deficit that existed in Sample 2.

Fig. 1 presents in graphic form the bicarbonate deficit which was found in 1 experiment. This experiment is not one of the 7 on which complete electrolyte data were obtained. The dotted line represents the CO_2 absorption curve for blood from this animal as calculated from hemoglobin concentration. The solid line is the increase in the bicarbonate concentration as actually obtained when the dog breathed first 30 per cent carbon dioxide (pH 6.8) and

later 40 per cent carbon dioxide (pH 6.5). The point at pH 7.15 is that obtained on the blood sample drawn after the dog had been returned to breathing air. In this particular experiment, in order to obviate the effect of another anesthetic agent, the dog was anesthetized with carbon dioxide. It is obvious that a considerable bicarbonate deficit existed in all of the samples of blood drawn after the control sample. The calculated deficit amounted to about 14 mEq.

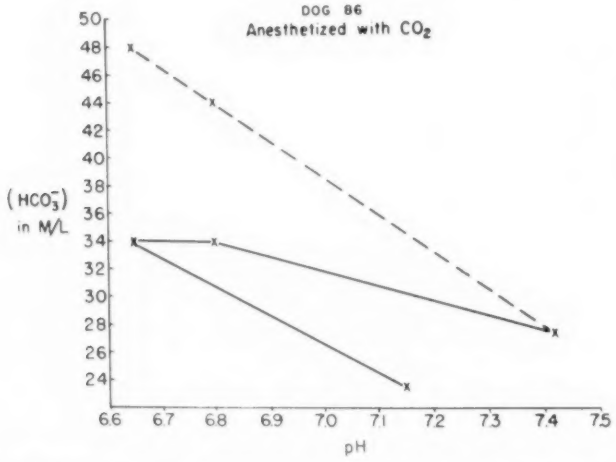

Fig. 1.—Beginning with the point at pH 7.42 the points connected by the solid line are values obtained for plasma bicarbonate and pH with the dog breathing successively 30 per cent CO_2 for 2 hours, 40 per cent CO_2 for 2 hours, and air for 5 minutes. The dashed line is the calculated buffer curve for the blood of this dog.

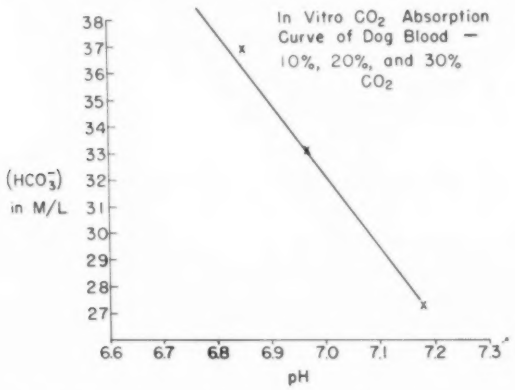

Fig. 2.—Graph showing the agreement between calculated buffer curve (solid line) and values obtained by equilibration with different CO_2 tensions.

per liter when the dog had been returned to breathing air. Unfortunately, none of the 7 animals in which complete electrolyte studies were made showed this degree of bicarbonate deficit, particularly after they had been returned to breathing air. In order to be sure that the slope of the calculated absorption curve was not fictitiously high, in vitro experiments were carried out. The results of one of these experiments is shown in Fig. 2. The solid line is the calculated

absorption curve placed at the bicarbonate and pH value obtained on a sample of blood equilibrated with 10 per cent CO_2. The other 2 points were those obtained on samples of blood equilibrated with 20 and 30 per cent CO_2, respectively. It is obvious that the calculated absorption curve is in reality the curve which is obtained on dog blood equilibrated with different CO_2 mixtures in vitro.

Table IV presents results of electrolyte analyses on 2 in vitro experiments carried out on dog blood. In each case the blood was divided into 2 aliquots; 1 sample was equilibrated with 10 per cent CO_2 and 90 per cent O_2 and the second sample with 30 per cent CO_2 and 70 per cent O_2. No appreciable bicarbonate deficit was present in either of these samples after equilibration with 30 per cent CO_2. Several differences between the results obtained on these blood samples and the in vivo experiments are evident. No change in hemoglobin concentration, protein concentration, potassium, or calcium and magnesium concentrations took place. The protein decrease expressed as milliequivalents per liter is entirely due to the decrease in pH. Chloride concentration decreased, sodium concentration regularly increased, and the increase in bicarbonate concentration is the result of the decrease in chloride and protein and the increase in sodium.

DISCUSSION

The appearance of what is commonly called metabolic acidosis during severe respiratory acidosis is puzzling. This metabolic acidosis must be due either to an increase in anions other than bicarbonate or a decrease in cations or some combination of the two. The result of either or both of these changes is a decrease in the HCO_3^- concentration below that which should be present at the existing pH and P_{CO_2}. What is actually measured then is a bicarbonate deficit; it seems to me that this term has more meaning than the term metabolic acidosis. In 1950, Beecher and Murphy[2] reported bicarbonate deficits as large as 9.3 mM. per liter during thoracic operations in which CO_2 accumulated to tensions of 100 mm. Hg with pH values as low as 7.09. This was puzzling in view of the fact that ether anesthesia alone does not produce such a metabolic acidosis.

At about this time we were observing similar bicarbonate deficits in dogs subjected to 3 to 4 hours of breathing mixtures of 15 to 30 per cent carbon dioxide. This deficit in bicarbonate is precisely opposite to the change which should occur in order to accomplish compensation for respiratory acidosis. That this amounted to an appreciable decrease in bicarbonate concentration in some cases can be seen in Fig. 1. By an analysis of all of the cations and of 4 of the anions we hoped to be able to find the changes in plasma composition which were responsible for this bicarbonate deficit. It was hoped that 1 or 2 animals in the series would have bicarbonate deficits of 10 to 12 mEq. per liter still present in the third blood sample, which was drawn after return to breathing air. With comparable pH's on Samples 1 and 3 in such a case, errors in the calculation of binding power of protein and phosphate and errors in correcting bicarbonate concentration to the value it would have at pH 7.4 would be minimal. Comparison of the electrolyte composition of 2 such samples of blood should indicate

the changes responsible for the bicarbonate deficit. Unfortunately such results were not obtained on any of the 7 experiments run. The largest deficit which still existed when Sample 3 was drawn was in Experiment 7 (Table IV). The combined errors of 7 determinations and 3 calculations could easily obscure differences of 4 to 6 mEq. per liter.

The calculated deficit on Sample 2 which averaged between 11 and 14.5 mEq. per liter should certainly be detectable in concentrations of other ions in this sample as compared with Sample 1. In this instance, however, we are not attempting to find why the bicarbonate concentration is less than it was in Sample 1, but why, in spite of an increase, it has increased less than it should have. This is more difficult. Since in vitro equilibration of blood samples with high CO_2 concentrations does result in the expected plasma bicarbonate concentration, a comparison of the electrolyte composition of such equilibrated samples with those obtained in vivo should give some information on the bicarbonate deficit observed in the in vivo experiments. In the animal experiments, inorganic phosphate concentration regularly increased during acidosis. The mean increase amounted to about 3 mEq. per liter. In the case of the equilibrated blood samples, the calculated phosphate concentration, expressed as milliequivalents per liter of phosphorus, decreased 0.5 mEq. per liter. Sodium increased in both types of experiments; however, the increase in the in vitro experiments was considerably greater than that observed in the animal experiments. In not one of the 7 animal experiments was the increase in sodium concentration as great as it was in either of the in vitro experiments. The increase in potassium concentration in the animal experiments is still not sufficient to offset the greater increase in sodium concentration in the in vitro experiments. It would appear then that the deficit in bicarbonate concentration in Sample 2 is due to the rise in phosphate concentration and the smaller increase in sodium concentration as compared with the in vitro experiments. It should be pointed out that a comparison between Sample 2 on the animal experiments and the 30 per cent CO_2 equilibrated sample in the in vitro experiments is not entirely valid since the blood in Sample 2 from the animal experiments is in equilibrium with a somewhat higher tension of CO_2. There is no evidence in any of the data presented in this study of an increase in the R anions. In an earlier investigation we analyzed plasma for lactic acid concentration and found no evidence of an increase during the severe respiratory acidosis. Other constituents of this fraction have not been assayed.

The difference between total cations and total anions (R) was routinely less than that which is usually reported. This appears to be due, in part at least, to the fact that our chloride values were high. If there is a consistent error here, however, it would not invalidate the attempt to find a change between Samples 1 and 2 or 1 and 3 which would account for a bicarbonate deficit.

The large variation in P_{CO_2} on Sample 2 in the animal experiments may be partially accounted for by the fact that any error in pH under these conditions produces a larger change in calculated P_{CO_2}. For example, a difference of 0.05 pH results in a 10 per cent change in P_{CO_2} whether the total P_{CO_2} is 250 or 40 mm. Hg. However, the influence of this variation in CO_2 tension on

the bicarbonate concentration is not great. A second factor responsible for the variation in CO_2 tension on Sample 2 is the inability to maintain exactly 40 per cent carbon dioxide in the inhaled mixture.

SUMMARY

Seven dogs breathed 30 per cent CO_2 and 70 per cent O_2 for 2 hours after which they breathed 40 per cent CO_2 and 60 per cent O_2 for an additional 2 hours. Arterial blood samples were drawn before, at the termination of breathing CO_2, and 15 minutes after the termination of breathing CO_2. Hemoglobin and pH determinations were made on blood; sodium, potassium, calcium and magnesium, bicarbonate, chloride, protein, and phosphate determinations were made on plasma. A significant bicarbonate deficit was calculated to be present in all of the blood samples drawn at the termination of breathing CO_2. After 15 minutes of breathing air this deficit was negligible in 5 of 7 experiments. In 2 in vitro experiments, aliquots of dog blood were equilibrated with 10 per cent CO_2 and with 30 per cent CO_2; the same analyses were carried out on the blood and plasma from these samples as were made in the in vivo experiments. No appreciable bicarbonate deficit was found in these experiments. Comparisons of the data in the 2 sets of experiments indicate that the bicarbonate deficit in the in vivo situation is due to an increase in phosphate concentration and to a smaller increase in sodium concentration during acidosis than was present in the in vitro samples. No evidence was found that the R fraction, which includes organic acid anions, increased during respiratory acidosis in these experiments.

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SIMULTANEOUS MEASUREMENT OF PLASMA VOLUME, EXTRACELLULAR FLUID VOLUME, AND RED BLOOD CELL MASS IN MAN UTILIZING I^{131}, $S^{35}O_4$, AND CR^{51}

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THE determination of the nature of fluids and electrolytes and their distribution in the body has become increasingly important in the care of acutely and chronically ill patients. The compartmentalization and rapid determination of the various body fluids have been made more accurate and feasible by the recent widespread use and availability of radioisotope tracer methods. As with most biologic systems, methods for measuring net change, or rates of change, of fluids and ions across cell membranes are more pertinent if as many variables as possible can be measured simultaneously.

Isotope dilution techniques, in which radioactive iodinated serum albumin (RISA) is used to measure the plasma volume, have been shown repeatedly to be valid procedures under most circumstances.[1-5] Similarly, the tagging of erythrocytes with chromate^{51} has proved to be an efficient and rapid method for measuring the total red blood cell mass in the body.[6-9] In addition, Gurney and Bolt[10] have devised a method for the measurement of plasma volume and red blood cell mass in which a combination of I^{131}-tagged human serum albumin and chromate^{51}-labeled erythrocytes are given in 2 successive injections. The use of radioactive sulfate labeled with sulfur^{35} has been shown to be a convenient method for rapid determination of the volume of extracellular fluid in man and in dogs by Walser and co-workers.[11] These investigators have shown the obvious advantages of the rapid dilution radioisotope tracer techniques in overcoming such problems as (1) lengthy time for equilibration of various dyes, (2) administration of large amounts of ions which might conceivably change the diffusion rates across body cell membranes, (3) elimination of laborious chemical procedures, and (4) accurate reproducibility.

The purpose of this article is to describe a method for the simultaneous measurement of plasma volume, red blood cell mass, and extracellular fluid volume in which radioactive iodinated serum albumin, chromate^{51}-tagged red cells, and S^{35}-tagged sulfate are used. The method has been so devised that this can be accomplished with a single intravenous injection of a combination of isotopes; all measurements can be made from a single venous blood sample withdrawn approximately 20 minutes after the injection. This is done by using a

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combination of radiation detection instruments[12, 13] which will differentiate the energy emission of the 3 simultaneously administered isotopes on a single mixed venous blood sample.

MATERIALS AND METHODS

1. *Materials.*—(a) Carrier-free S^{35} in the form of H_2SO_4 in weak hydrochloric acid* was prepared for injection by dilution of an appropriate volume with normal saline after it had been neutralized with 2N sodium hydroxide. A final concentration of 100 μc per milliliter was obtained and the solution was autoclaved. (b) Sterile radioactive iodinated (I^{131}) serum albumin† was diluted in normal saline to give a concentration of 10 μc I^{131} per milliliter. (c) The chromate was obtained as sterile radioactive chromate^{51}† in the form of chromate (Rachromate); this was diluted to give a concentration of 100 μc per milliliter.

Radioactive (S^{35}) sulfate is a weak beta emitter with a half-life of 87.1 days. The chromate^{51} is predominately a gamma emitter with a half-life of 27.8 days. Radioactive iodinated serum albumin is also predominately a gamma emitter with a half-life of 8.14 days, although it emits a gamma ray of different wavelength from that of chromate.

2. *Procedure for Injection.*—One milliliter of the saline dilution of the sodium sulfate with S^{35} is used for the sulfate injection (100 μc). One milliliter of radioactive iodinated serum albumin (10 μc) is mixed in the same syringe. The radioactive chromate-tagged cells are prepared by mixing 20 ml. of subject's whole blood with 7 ml. of modified acid-citrate-dextrose solution† in a 50 ml. vial: 150 μc radioactive sodium chromate is added; the mixture is incubated at 37° C. in a water bath for 20 minutes with occasional agitation; 50 mg. of ascorbic acid contained in 1 ml. is added, thus converting the extracellular chromate to trivalent chromic ion, after which no further erythrocyte tagging occurs. After gentle agitation, 10 ml. of this mixture is added to the syringe containing sulfate and iodinated serum albumin, and the entire mixture is injected intravenously over a period of 1 minute. Care is taken to maintain sterile conditions throughout the preparation of the sample to be injected.

3. *Samples* for radioactive counting and hematocrits were obtained through a peripheral vein by the use of a small polyethylene catheter introduced through an intravenous needle in the opposite arm from which the isotopes were injected. The catheter was inserted through the antecubital vein approximately to the level of the subclavian vein so that free flowing venous samples could be obtained at all times. When this procedure was not possible, samples were obtained from the peripheral vein in the antecubital area.

4. *Preparation of plasma and whole blood for counting* was done in the following manner:

A. *Radioactive iodinated serum albumin:* (1) The plasma samples were obtained by centrifuging whole blood specimens for 30 minutes at 2,000 g (3,700 r.p.m.); (2) 0.5 ml. samples of undiluted plasma were then mounted on individual aluminum planchets and allowed to evaporate at room temperature for counting in a flow chamber type detection instrument with a micromil window (Nuclear model D-47); and (3) undiluted plasma (0.5 ml.) was similarly prepared for counting in a liquid well type scintillation chamber attached to a Packard energy differentiating instrument.

B. *Chromate:* (1) Plasma was obtained as described and 0.5 ml. was mounted in undiluted liquid form for counting in the scintillation type chamber attached to a Packard energy differentiating instrument; (2) 0.5 ml. of undiluted plasma was mounted on an aluminum planchet and allowed to dry at room temperature for counting in a flow chamber as described; and (3) samples for counting whole blood chromate were prepared in 0.5 ml. undiluted liquid form for counting in the well chamber. No dry planchet counts were necessary for whole blood counting of chromate. All whole blood samples must be prepared before plasma is removed for plasma samples.

*Obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.
†Obtained from Abbott Laboratories, North Chicago, Ill.

C. *Sulfate:* (1) Plasma was obtained in the same fashion, and 0.5 ml. of undiluted plasma was similarly mounted on an aluminum planchet and allowed to dry at room temperature for flow chamber counting; and (2) 0.5 ml. plasma samples were mounted in test tubes for scintillation chamber counting. All plasma and blood samples were counted in duplicate for a minimum of 5,000 counts.

5. *Standards* were prepared and counted in duplicate. The sulfate standard was prepared as 1:1000 saline dilution of the amount injected (approximately 100 μc of S^{35} activity). This standard was then counted both in the saline dilution and mixed with different volumes of plasma. This was done in order to correct for any plasma correction factor which could possibly be present in the plasma samples. The I^{131} standard was made in a similar manner in the same dilutions and counted with and without added plasma both in the Packard counter and the flow counter. In addition to the sulfate and I^{131} standards, plasma and whole blood chromate standards were made up from the unused portion of the withdrawn blood which had been tagged with chromate^{51}. This specimen was made up as 1:25 dilution of the whole blood. The sample was then centrifuged and 1:10 dilution of the plasma was similarly made. These samples were then counted in duplicate on both flow counter and the Packard counter.

6. *Urine samples* were collected during the period of equilibration. These samples were mounted both in liquid form for scintillation counting and dry form in aluminum planchets for flow counter counting.

CALCULATIONS

The erythrocyte mass was determined using the principle of isotope dilution. A measured volume of whole blood with approximately 95 per cent of the erythrocytes tagged with Cr^{51} is injected and allowed to equilibrate in the body for a period determined by obtaining serial blood samples (usually 20 minutes) at 10 second intervals. Since 3 different radioactive materials were injected simultaneously, all but the Cr^{51} tagged to the erythrocytes must be eliminated for the calculation of the erythrocyte mass. The weak beta emitter (S^{35}) can be effectively screened from the gamma emitters (Cr^{51} and I^{131}) by a procedure which utilizes a well type scintillation chamber. This leaves 2 gamma fractions to be separated: I^{131} gamma counts which were injected as RISA and plasma chromate^{51} ions that were converted to chromic^{51} after tagging and were injected as untagged plasma chromic^{51}. These separations can be done with the use of a type of energy differentiating monitor with a well type scintillation pickup chamber attached. Two electrical window openings on the Packard instrument which will effectively differentiate the gamma radiation caused by chromate and chromic from that caused by I^{131} are determined using standard solutions. This particular reading for our instrument was a tap voltage of 850 and electrical windows set at 10-24-28 and 10-30-35. The 10-24-28 was a chromate-sensitive zone which allowed a maximum number of chromate counts to be recorded whereas only 30 per cent of the I^{131} counts which were present at the iodine-sensitive window (10-30-35) were recorded. At the 10-30-35 window a maximum number of iodine counts would appear (approximately 3 times the number of I^{131} counts which appeared at the chromate-sensitive [10-24-28] zone), and chromate was effectively screened to almost zero.

Standards of I^{131} and chromate^{51} were then counted at various dilutions above and below those expected in the plasma and in the working standards. The ratio of that isotope counted at the 2 windows selected was found to be constant for iodine and chromate at the different dilutions, which was an average ratio of 0.307 I^{131} at 10-24-28: I^{131} at 10-30-35. It was learned that this determination should be made with each patient or particular time that the machine is used in view of the fact that occasionally the electrical sensitivity of the machines would change; consequently, the ratio used in the calculations may vary from patient to patient.

1. *Red Blood Cell Mass.*—The following formulae were used:

(1) Total whole blood (0.5 ml.) liquid sample counts at the iodine-sensitive zone (10-30-35) *minus* the background of the Packard instrument per minute *times* the ratio of

counts caused by $\frac{I^{131} \text{ standard at } 10\text{-}24\text{-}28}{I^{131} \text{ standard at } 10\text{-}30\text{-}35}$ *equals* the I^{131} counts which will appear at the 10-24-28 zone.

(2) Then total whole blood 0.5 ml. liquid sample counts at the 10-24-28 zone (which is the chromate-sensitive zone) *minus* the background of the Packard instrument *minus* the counts obtained from Formula (1) *equals* the actual chromate counts in the whole blood sample.

Technically, an extremely small fraction of chromate^{51} appeared at the I^{131}-sensitive zone, since it was impossible to select a window which would completely screen the chromate count and still allow a sufficient number of iodine counts to be left present for accurate counting. In order to correct for this small fraction accurately the following equations were developed:

T_2 = Total counts in whole blood samples at 10-24-28 (Cr-sensitive zone).
T_3 = Total counts in whole blood sample at 10-30-35 (I-sensitive zone).
I_2^{131} = I^{131} counts in the sample at 10-24-28.
I_3^{131} = I^{131} counts in the sample at 10-30-35.
Cr_2^{51} = Cr^{51} counts in the sample at 10-24-28.
Cr_3^{51} = Cr^{51} counts in the sample at 10-30-35.

$$K_1 = \frac{I^{131} \text{ standard at } 10\text{-}24\text{-}28}{I^{131} \text{ standard at } 10\text{-}30\text{-}35}$$

$$K_2 = \frac{Cr^{51} \text{ standard at } 10\text{-}30\text{-}35}{Cr^{51} \text{ standard at } 10\text{-}24\text{-}28}$$

$$Cr_3^{51} = K_2(T_2 - I_2^{131}) \quad (1)$$
$$I_2^{131} = K_1(T_3 - Cr_3^{51}) \quad (2)$$
$$Cr_2^{51} = K_2Cr_2^{51} \quad (3)$$
$$I_3^{131} = T_3 - Cr_3^{51} \quad (4)$$

or

$$Cr_3^{51} = T_3 - I_3^{131}$$

Rearrange Equation (1):

$$Cr_3^{51} = K_2T_2 - K_2I_2^{131}$$
$$K_2I_2^{131} = K_2T_2 - Cr_3^{51}$$
$$I_2^{131} = \frac{K_2T_2 - Cr_3^{51}}{K_2} \quad (5)$$

Substitute Equation (2):

$$K_1T_3 - K_1Cr_3^{51} = \frac{K_2T_2 - Cr_3^{51}}{K_2} \quad (6)$$

Substitute Equation (3) and solve for Cr_2^{51}:

$$K_1T_3 - K_1K_2Cr_2^{51} = \frac{K_2T_2 - K_2Cr_2^{51}}{K_2}$$
$$Cr_2^{51} - K_1K_2Cr_2^{51} = T_2 - K_1T_3$$
$$Cr_2^{51}(1 - K_1K_2) = T_2 - K_1T_3$$
$$Cr_2^{51} = \frac{T_2 - K_1T_3}{(1 - K_1K_2)} \quad \text{(Total chromate in whole blood.)}$$
$$Cr_2^{51} = \frac{T_2 - K_1T_3}{1 - K_1K_2}$$

Therefore Formula (1) = K_1T_3
Formula (2) = $T_2 - K_1T_3$
Formula (3) = $\frac{T_2 - K_1T_3}{1 - K_1K_2}$

Correction Factor = $1 - K_1K_2$

Substituting 0.306 (determined) for K_1 and 0.04 (determined) for K_2, the chromate^{51} counts appearing at 10-24-28 zone will equal

$$Cr_2^{51} = \frac{T_2 - 0.306\ T_3}{1 - 0.306 \times 0.04} = \frac{T_2 - 0.306\ T_3}{0.98776}$$

To solve for Cr_2^{51} PL (plasma), substitute K_3 for K_2 and use *plasma* counts where

$$K_3 = \frac{\text{Plasma } Cr^{51} \text{ standard at 10-30-35}}{\text{Plasma } Cr^{51} \text{ standard at 10-24-28}}$$

Formula (4) $Cr_{2PL}^{51} = \frac{T_2 - K_1T_3}{1 - K_1K_3}$

This small fraction would be constant and, therefore, would not interfere even if the window sensitivity of any given energy differentiating counter could not be approximated as closely as the particular instrument on which these counts were made, which was consistently less than 2 per cent.

(3) Therefore, the final corrected chromate^{51} counts in whole blood equal the final figure in Formula (2) divided by $1 - K_1 \times K_2$.

(4) To remove the gamma counts caused by chromate^{51}, which was injected in plasma as chromic^{51} (not attached to red cells), the same counting and calculations as outlined in Formulas (1), (2), and (3) are done for *plasma* and are subtracted from final Formula (3) in order to obtain only the chromate^{51} counts in whole blood sample which are attached to the red blood cells.

The red blood cell mass is then calculated by the following formula:

(a) Activity within the tagged erythrocytes injected: milliliters Cr^{51}-labeled blood injected times [chromate activity per milliliter whole blood at 10-24-28 zone minus (chromate activity per milliliter plasma at 10-24-28 zone) times (1 minus hematocrit)]. These measurements (a) are all done on Cr^{51}-tagged blood standard which remains after removal of amount necessary for injection.

(b) Activity of 1 ml. of erythrocytes from venous blood sample after complete mixing: chromate activity per milliliter of blood [Formula (3)] minus [chromate activity per milliliter plasma (Formula (4)) times (1 minus hematocrit)] divided by hematocrit.
These values (b) are all obtained from the blood sample drawn 20 minutes after injection of all isotopes.

(c) (a) activity within tagged radiochromate injected divided by (b) activity of 1 ml. of erythrocytes after complete mixing equals erythrocyte volume.

2. *Plasma Volume.*—Again using the isotope dilution principle, plasma volume can be calculated by dividing radioactivity injected (in counts per minute) by the radioactivity of 1 ml. of plasma (in counts per minute), obtained after complete mixing of the radioactive material (I^{131}).

Total gamma counts injected are obtained by counting 1 ml. of a 1:1,000 dilution of RISA in the Packard energy differentiating instrument at the 10-30-35 zone.

The plasma gamma counts are due predominantly to I^{131}; however, some of the plasma gamma counts will be due to the chromic^{51} ions which were not tagged to red cells. Consequently, the gamma counts caused by these 2 ions must be separated; this is done in the following manner: the I^{131} standard, the chromate^{51} plasma standard, and 0.5 ml. of liquid plasma sample are counted in the Packard counter at both the iodine^{131}-sensitive zone (10-30-35) and at the chromate^{51}-sensitive zone (10-24-28). By using *plasma* instead of whole blood, the total *plasma* counts at 10-30-35 *minus* the background of the instrument in counts per minute *minus* (the total *plasma* counts at 10-24-28 *times* the ratio of the plasma chromate$_3^{51}$ standard at 10-30-35 *divided by* the plasma chromate$_2^{51}$ standard at 10-24-28 *equals* iodine counts in plasma at 10-30-35 zone.

Solve for plasma I_3^{131}: substitute Equation (4) in Equation (6); and substitute K_3 for K_2: (Total counts are from plasma samples.)

$$K_1T_2 - K_1Cr_3^{51} = \frac{K_2T_2 - Cr_3^{51}}{K_2} \qquad (6)$$

$$Cr_3^{51} = T_3 - I_3^{131} \qquad (4)$$

$$K_1T_3 - K_1(T_3 - I_3^{131}) = \frac{K_3T_2 - (T_3 - I^{131})}{K_3}$$

$$K_1T_3 - K_1T_3 + K_1I_3^{131} = \frac{K_3T_2 - T_3 + I_3^{131}}{K_3}$$

$$K_3K_1I_3^{131} = K_3T_2 - T_3 + I_3^{131}$$

$$K_3K_1I_3^{131} - I_3^{131} = K_3T_2 - T_3$$

$$I_3^{131}\ (K_3K_1 - 1) = K_3T_2 - T_3$$

$$I_3^{131} = \frac{K_3T_2 - T_3}{K_3K_1 - 1} = \frac{T_3 - K_3T_2}{1 - K_3K_1}$$

Correction for the I^{131} which appears at the chromate-sensitive 10-24-28 window will be $T_3 - K_3 \times T_2 \div 1 - K_1 \times K_3$. Plasma volume in milliliters is the total counts of I^{131} injected $\div (T_3 - K_3 \times T_2 \div 1 - K_1K_3)$, which is the concentration of counts per milliliter in plasma caused by I^{131}.

3. *The Determination of the Extracellular Fluid Volume.*—Still utilizing the principle of isotope dilution, the extracellular fluid volume is measured by the radioactive S^{35}-tagged sulfate which has been shown to be freely diffusible in extracellular fluid. The actual volume is then measured by the formula: total counts of $S^{35}O_4$ injected *minus* the counts excreted during equilibration time *divided by* the concentration in the extracellular water. This equilibration measures the total extracellular fluid, both interstitial and intravascular extracellular fluid.

The $S^{35}O_4$ counts were obtained in the following fashion:

1. Standards of S^{35}, I^{131}, and Cr^{51} were counted in a micromil window flow counter which counts both beta and gamma radiation, and also in a scintillation chamber with energy differentiating counter which does not count beta particles. Counting all 3 standards, all S^{35} beta counts were removed by counting in the scintillation chamber; consequently, the difference in the total counts obtained on the window counter minus the counts caused by gamma radiation which were obtained from the Packard equal the beta counts present in the standard. These were due to the beta emitter, S^{35}.

2. In order to determine the counts as gamma counts from iodine and chromate which would appear in the window in a plasma sample, a ratio of standards for the I^{131} and Cr^{51} in the Packard to those present in the window counter is determined by counting these standards on both instruments. Knowing the counts expected to appear in the window from this ratio, the total counts which actually appear minus the expected counts caused by I^{131} and Cr^{51} equal the beta counts actually present in the unknown sample caused by $S^{35}O_4$. Using the actual counts measured caused by I^{131} and Cr^{51} in the Packard energy differentiating counter as outlined previously for any individual sample, the number of counts expected to appear in the window counter, as predicted from the Packard (gamma) count present in the sample, is then subtracted from the total counts actually obtained in the window counter. These represent beta counts caused by $S^{35}O_4$.

3. The final calculations of extracellular fluid are then corrected for Donnan factor of 0.95 and for water content of serum assumed to be 93 per cent.[11] In addition, the urinary loss of beta counts was actually measured, as urine was collected during the period of equilibration. These total counts are then subtracted from the counts injected in order to obtain accurately the amount of injected radioactivity which was diluted and measured in the plasma sample drawn at 20 minutes after the injection.

RESULTS

After preliminary studies and verification in animals, the method described was subsequently applied to normal human subjects. In Table I will be seen the results of the simultaneous determination of plasma volume, red blood cell volume, and extracellular fluid volume in 9 normal adults. It will be seen that the plasma volume agrees closely with findings of others.[1-5] Also the total red

blood cell mass agrees with the determination of others.[6-10] The extracellular fluid volume can be seen to agree with the previously determined extracellular fluid volume by this method in man.[11] It should be further pointed out that the variation in the extracellular fluid volume expressed as per cent of body weight was probably a function of variation in total lean body mass. The total body extracellular fluid varied from 18.8 to 24.7 per cent of body weight. The mean figure was 21.64 per cent of body weight.

TABLE I

SUBJECT (NO.)	AGE (YRS.)	SEX	WEIGHT (KG.)	PLASMA VOLUME (ML.)	R.B.C. VOLUME (ML.)	ECF* VOLUME (ML.)	TOTAL BLOOD VOLUME (ML.)	BODY HEMATOCRIT	PERIPHERAL HEMATOCRIT
1.	28	M	90	2,123	1,255	21,050	3,378	37.2	40.0
2.	32	F	55	2,523	1,627	10,925	4,151	39.2	43.1
3.	40	F	101	2,400	1,835	24,084	4,235	43.3	43.0
4.	68	M	76	2,777	1,748	18,776	4,525	38.6	41.0
5.	64	M	66	2,344	1,110	15,248	3,454	32.1	34.0
6.	37	F	60	2,328	1,153	11,278	3,481	33.1	35.9
7.	65	F	60	2,636	1,253	11,315	3,289	38.1	40.3
8.	60	F	75	2,666	1,042	17,532	3,708	28.0	33.0
9.	40	F	60	2,074	1,194	11,563	3,268	36.0	36.5

*Extracellular fluid.

As pointed out by Gurney and Bolt, the body hematocrit as determined by the ratio of red cell mass to total blood volume, as measured by the combination of red blood cell volume and plasma volume by the Cr^{51} and I^{131} method, probably gives a more accurate total circulating average hematocrit than the peripheral hematocrit.[8, 14] This is particularly true under abnormal circumstances. Measured erythrocyte and plasma volumes correlate quite closely in normal resting subjects (Table I) in all 10 instances. The total body hematocrit in every instance is slightly lower than the actually measured peripheral hematocrit.

SUMMARY

A method is described for the simultaneous measurement of plasma volume, red blood cell mass, and extracellular fluid volume, in which radioactive iodinated serum albumin (I^{131}), chromate^{51}-tagged red blood cells, and sulfur^{35}-tagged sulfate are used. The method has been so devised that it can be accomplished with a single intravenous injection using a combination of isotopes of I^{131}, Cr^{51}, and S^{35}; all determinations can be made from a single venous blood sample withdrawn approximately 20 minutes after the injection. This is accomplished by using a combination of radiation detection instruments which will differentiate the energy emission of the 3 simultaneously administered isotopes in a single mixed sample.

Data are presented on 9 normal patients in whom the method was applied. The results agree closely with those of plasma volume, red blood cell mass, and extracellular fluid volume as measured individually by various methods in other normal patients.

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LABORATORY METHODS

SPECTROPHOTOMETRIC DETERMINATION OF BLOOD OXYGEN CONTENT

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THE multiplicity of samples encountered in cardiac catheterization and certain experimental procedures has emphasized the need for a rapid, quantitative determination of the blood oxygen content to replace the time-consuming gasometric methods. Spectrophotometric methods based on the wide difference in light absorption of reduced and oxyhemoglobin at certain wavelengths are now in extensive use.[1-11] Most of these methods are suitable for human and animal studies not complicated by rapid changes in the total hemoglobin level.

In this report, a direct spectrophotometric method is presented that is capable of excellent replication and is useful in the open chest animal preparation despite the complications of an auxilliary pumping device and attendant shifts in hemoglobin concentration. It may also be applicable in connection with surgical procedures in man, especially those which involve the extracorporeal pump.

MATERIALS AND METHODS

Light absorption of anaerobically handled, hemolyzed dog blood is determined in a silica cell of 1 mm. light path at a wavelength of high absorbancy (655 mμ) for reduced hemoglobin† and relative insensitivity to HbO_2. T-Hb is determined separately to avoid alteration of the wavelength setting. A multiple regression relation dependent on light absorption at the selected wavelength and the concentration of T-Hb was derived.

Sampling and Procedure.—Every precaution is taken to avoid the trapping of air in blood samples. Syringes used to collect the blood and to hemolyze the blood were treated with silicone, lightly greased, and provided with a small amount of mercury to fill the dead space. A 50 per cent solution of saponin (Kodak) is used as the hemolyzing agent. The

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†For convenience, the following symbols will be used: HbO_2 for oxyhemoglobin, Hb for reduced hemoglobin, T-Hb for total hemoglobin, MHb for methemoglobin, HbCO for carboxyhemoglobin, S-Hb for sulfhemoglobin, CNMHb for cyanmethemoglobin, E_r for extinction coefficient for reduced hemoglobin at 655 mμ, E_o for extinction coefficient for oxyhemoglobin at 655 mμ, and O.D. for optical density.

freshly prepared solution is filtered while hot and drawn in a 2 ml. greased syringe fitted with a 26 gauge needle. The shaft of the needle is equipped with a ½ inch rubber sleeve to ensure a seal when saponin is transferred from the dispensing syringe to the blood hemolyzing syringe. A volume of 0.04 ml. of saponin per 1.0 ml. of blood is dispensed with a micrometer burette assembly.*

The blood collecting syringe is rotated to mix the blood thoroughly. The syringe cap is then replaced by a 20 gauge, short, blunt needle fitted with a rubber sleeve. A 1.0 ml. sample of blood is expelled through the needle and delivered into the tip of a 2.0 ml. (nontuberculin) syringe prepared as described. The 2.0 ml. syringe is attached to the burette assembly and the saponin is introduced.

With the addition of saponin the syringe is capped and shaken. The mercury in the syringe facilitates mixing and rapid hemolysis. The cap of the syringe is then replaced by a 5 cm. polyethylene tube (inside diameter 1.5 mm.) fitted with a rubber sleeve for snug coupling with the syringe tip. Hemolyzed blood is transferred to a 1.0 cm. Beckman silica cell containing a polished glass prism† that reduces the light path to 1.0 mm. The optical density is read on a Beckman DU spectrophotometer at 655 mμ and a slit width of 0.01 mm. This slit width and wavelength setting require the use of the photomultiplier attachment.

The T-Hb is determined by diluting 0.1 ml. of whole blood to 25 ml. with cyanmethemoglobin reagent prepared according to Walter Reed Army Institute of Research.[12] This procedure converts all hemoglobin moities to CNMHb which has an absorption peak at 540 mμ. The determination of T-Hb by this method obviates the need to change the wavelength setting for each determination. Still another advantage is the greater light path (1 cm.) that may be used for T-Hb analysis.

Determination of Extinction Coefficients and Wavelength.—The extinction coefficients HbO_2 at 655 mμ, E_o, and Hb at 655 mμ, E_r, were obtained on hemolyzed samples of completely oxygenated blood. Blood samples were reduced by one of 2 procedures: addition of sodium hydrosulfite crystals to the blood in the cuvette and reduction in the Van Slyke manometric chamber under reduced pressure and the addition of much less sodium hydrosulfite. In both instances the samples were read immediately after reduction.

Optical density, O.D., readings were taken for buffered solutions (dilution 1:50 in phosphate buffer, ionic strength 0.1 at pH 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0) of Hb and MHb at 5 mμ wavelength intervals between 650 and 665 mμ. MHb is prepared by the addition of $K_3Fe(CN)_6$ to the buffered solutions. It was impossible to reproduce the wavelength setting at the "cross-over" point of Hb and MHb and this point was inferred from graphs of data obtained as described. In the procedure, 655 mμ was chosen rather than 657.5 mμ for the reason previously stated.

RESULTS AND DISCUSSION

Three factors determined the choice of 655 mμ as a suitable wavelength setting for the differential determination of Hb in the presence of HbO_2: (1) an easily reproduced wavelength setting of the monochromator, (2) a high ratio of the Hb to the HbO_2 molar extinction coefficients, and (3) a wavelength sufficiently close to the isobestic point of Hb and MHb so that the presence of small amounts of MHb would not yield a falsely high estimate of Hb. This setting yielded a ratio of Hb: HbO_2, 6.43 ± 0.09 (S.D. 0.26).

Table I shows the isobestic point of Hb and MHb as determined in 3 separate experiments. There is a characteristic dependency of MHb on pH.[1] The Hb is also affected by pH, and the net result of the pH dependency of both

*Micro-Metric Instrument Corp.
†Pyrocell Mfg. Co.

kinds of hemoglobin is a relatively constant "cross-over" or isobestic point throughout the pH range. The isobestic points derived from MHb and hemoglobin reduced with sodium hydrosulfite have a mean value close to 660 mμ confirming the findings of Gordy and Drabkin.[1] The isobestic points determined with hemoglobin reduced in the Van Slyke chamber after exhaustive evacuation and shaking are somewhat lower, 657.5 mμ. The Van Slyke reduction method is the only one that gave consistent reproducible O.D. readings.

TABLE I. DEPENDENCY OF THE HB ON PH OF SAMPLE IN RED SPECTRAL RANGE

	ISOBESTIC POINTS FOR HB AND MHB		
PH OF SAMPLE	REDUCTION IN $Na_2S_2O_4$	REDUCTION IN VAN SLYKE	
7.0	660.0	658.3	656.4
7.2	659.1	658.5	657.5
7.4	659.0	658.0	657.3
7.6	661.4	659.5	657.8
7.8	659.5	656.8	659.1
8.0	659.7	656.8	657.5
Mean	659.8 ± 0.4	657.5 ± 0.4	657.6 ± 0.1
S.D.	0.99	1.04	0.28

A short light path is required for the measurement of undiluted blood in the Beckman spectrophotometer.[3, 7] Our first attempts with the spectrophotometric method utilized an optical path of 0.1 mm. which yielded a range for O.D. between 0.002 to 0.090. As this is a relatively inaccurate region of the spectrophotometer, the light path was increased to 1.0 mm. Thereafter O.D. readings in the range of 0.100 to 0.500 were obtained.

Still another hindrance to replication of data was the need to shift the wavelength settings in order to read the sample at the isobestic point of Hb and HbO_2. To avoid this source of error the CNMHb method for the determination of T-Hb was routinely employed. Thus it became possible to leave the 655 mμ setting undisturbed during the interval needed to read a large series of samples. The CNMHb preparation of each sample was completed and read only after the entire series of readings at 655 mμ had been taken. Table II shows the reproducibility of the spectrophotometric readings at 655 mμ and a slit width of 0.01 mm. for 10 aliquots from each of 4 blood samples drawn from different dogs.

Perhaps the greatest obstacle to replication is the rapid change in O.D. that takes place between the addition of the hemolyzing agent and the measurement of O.D.[3, 6-8] Readings at 1 minute intervals over a period of 12 minutes elicited a 20 per cent elevation in O.D. Layering the sample with mineral oil or keeping the blood samples chilled before and after hemolysis did not prevent the rapid rise in O.D. readings. To minimize the error ascribed to this change, a 2 minute interval from the addition of the saponin to the spectrophotometric reading was found expedient and reproducible. This time factor was maintained for all analyses.

Ultimately, the relationship of the spectrophotometric readings at 655 mμ and the oxygen content is derived from the extinction coefficients of Hb, HbO_2,

and other forms of hemoglobin. Provided the T-Hb remains constant and no change in the concentration of the inactive hemoglobins (MHb, HbCO, and SHb) occurs, a linear relationship exists between the O.D. reading at 655 mμ and the oxygen content. A shift in the T-Hb concentration requires either a third axis or the use of a family of curves for graphic representation. Holling and coworkers[3] related the spectrophotometric readings on whole blood to oxygen saturation on the basis of a nomogram.

TABLE II. REPLICATION OF OPTICAL DENSITY FOR HbO_2 AT 655 mμ

	SERIES OF ANALYSES			
	1	2	3	4
	0.289	0.285	0.281	0.295
	0.285	0.275	0.275	0.286
	0.291	0.288	0.273	0.306
	0.300	0.289	0.276	0.287
	0.275	0.295	0.271	0.302
	0.295	0.302	0.278	0.292
	0.302	0.300	0.268	0.306
	0.304	0.296	0.265	0.291
	0.285	0.306	0.260	0.292
	0.295	0.306	0.262	0.300
Mean	0.292 ± 0.0024	0.294 ± 0.0030	0.271 ± 0.0023	0.296 ± 0.0024
S.D.	0.0072	0.0090	0.0070	0.0073

The oxygen content of a blood sample may also be calculated on the basis of the molar extinction coefficients of HbO_2 and Hb[1, 4, 5, 8] at an isobestic wavelength and at a nonisobestic wavelength. Another method for the determination of T-Hb, such as the CNMHb procedure, may be substituted for the isobestic determination of T-Hb. The millimolar concentration of T-Hb may then be calculated as follows:

$$O.D._{655} = (E_o \cdot d)\ (C_o) + (E_r \cdot d)\ (C_r) \tag{1}$$

$$C_o = \frac{O.D._{655}}{d \cdot E_o} - \frac{E_r}{E_o}\left(\frac{O.D._{540} \times \text{Dilution Factor}}{E_t} - C_o\right) \tag{2}$$

$$C_o = \frac{E_r}{(E_r - E_o)\,E_t} \cdot (O.D._{540} \times \text{Dilution Factor}) - \frac{O.D._{655}}{d \times (E_r - E_o)}$$

C_o and C_r are the concentrations of HbO_2 and Hb in millimoles per liter, d is the depth of the cell, E_r and E_o are the extinction coefficients of Hb and HbO_2 at 655 mμ, respectively, and E_t is the extinction coefficient of cyanmethemoglobin at 540 mμ. A satisfactory correlation (0.95, ±1.1 standard error of estimate) between Van Slyke oxygen content and the spectrophotometric procedure was obtained.

In the present study, a multiple regression equation based on the idea of a 3 dimensional plot of O.D. at 655 mμ, the blood oxygen content, and O.D. at 540 mμ for T-Hb is used. Blood samples were taken simultaneously from an artery, a vein, and a coronary vein of a dog. In a single animal this procedure was repeated at intervals 9 to 12 times. For a total of 88 blood samples, in 6 dogs, the following regression relationship was obtained as an estimating equation:

$$Y = 0.1090 - 2.744 \times 10^{-2}X_1 + 1.386X_2$$

Y is $O.D._{655}$, X_1 is the blood oxygen content, and X_2 is $O.D._{540}$.* The regression coefficients of the equation found for any single animal were not significantly different from those derived from pooling all observations. A coefficient of multiple determination of 0.8874 and a coefficient of multiple correlation of 0.9406 were obtained on the pooled analysis. Thus, about 12 per cent of the total variation around an ideal regression is not explained. The standard error of estimate based on the estimated and observed values of blood oxygen content is ±0.63 volume per cent.

The efficacy of the estimating equation was tested by comparing the blood oxygen content as determined by the Van Slyke method and by use of the estimating equation on 68 samples from 5 dogs (Fig. 1). These samples were independent of those used for the determination of the estimating equation. The coefficient of correlation for the estimated and observed values is 0.99 and the standard error of estimate ±0.62.

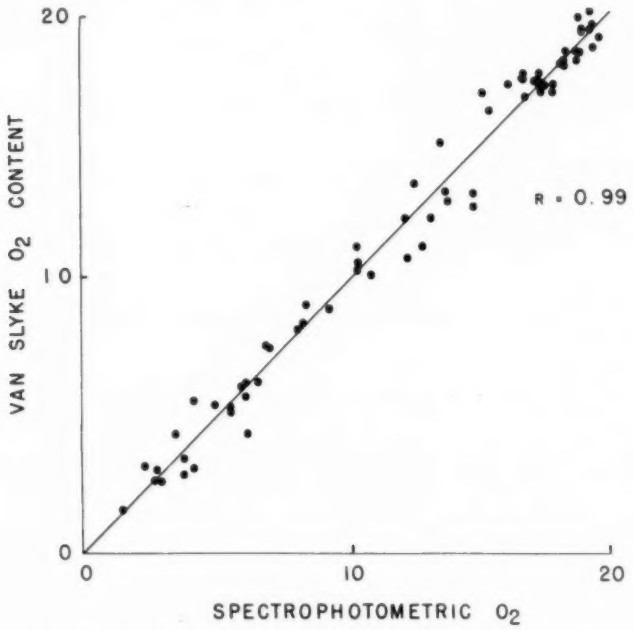

Fig. 1.—Graph showing relationship between values obtained in 68 analyses of blood oxygen by the Van Slyke manometric technique and by use of the estimating equation. The ordinate is the blood oxygen determined by the Van Slyke and the abscissa is that obtained by the estimating equation (both in volumes per cent).

SUMMARY

A quantitative method has been described for the determination of blood oxygen content. It involves the dependence of oxygen content on the spectral relation of oxy- and reduced hemoglobins and on the total hemoglobin concentration. The simple spectrophotometric procedure permits a prediction that

*In practice the regression relationship, derived as shown, may be restated as follows: Blood O_2 content = 39.72 − 36.44 ($O.D._{655}$) + 50.51 ($O.D._{540}$).

correlates closely with the measured oxygen content of a sample. The method is applicable and reliable, regardless of wide variations in total hemoglobin concentrations or extremely low oxygen capacities.

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THE PRESERVATION OF BLOOD FOR AMMONIA ANALYSIS

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THE concentration of free ammonium ion in blood (blood ammonia) has assumed increasing clinical interest recently because of its probable relationship to liver disease and hepatic coma. The measurement of this blood constituent was relatively inaccurate and cumbersome until Conway and Byrne[1] described their microdiffusion technique in 1933. Using this new method Conway[2] reported that the concentration of ammonia in blood increases if the blood is allowed to stand after venipuncture. He and, later, White and co-workers[6] stated that the circulating blood ammonia is normally zero or is immeasurable and then rises to the usual measured value during the first 5 minutes after sampling. This rapid initial increase has not been confirmed by us or by other investigators.[7, 8, 10, 11] All workers in this field, however, agree that significant ammonia production begins 15 to 20 minutes after shedding. This phenomenon imposes an inconvenient urgency in performing the test and might possibly deny the information to those who do not have the apparatus nearby. Many workers have tried different techniques to halt this evolution of ammonia in freshly drawn blood, in order to delay the performance of the test and still obtain accurate results. The purpose of this article is to present a simple and convenient method to preserve whole blood for ammonia analysis by quick freezing.

MATERIALS AND METHODS

The ammonium nitrogen determinations were done by McDermott's modification[5] of Conway's microdiffusion technique. The error of this method was found to be no greater than 10 per cent on recovery of ammonia from standard ammonium sulphate solutions. All subjects were volunteers with no historical or clinical evidence of liver disease. Samples were obtained by venipuncture with minimal stasis and were collected anaerobically in sealed heparinized syringes.

In Group I, blood from 5 subjects was used to assess the alleged increase in ammonia concentration during the first few minutes after shedding. The blood was drawn directly into a 10 ml. heparinized syringe, which was sealed and delivered into the diffusion dishes for analysis at 15, 30, 45, and 90 seconds after venipuncture. Blood from a patient with liver disease and known to have an elevated blood ammonia level was also used.

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In Group II, blood from 10 volunteers was used to determine the effect of quick freezing upon blood samples for ammonia analysis. From each subject a control sample was drawn into a 5 ml. heparinized syringe, which was immediately sealed, and analysis was performed 10 minutes after withdrawal. Through the same needle a 50 ml. heparinized syringe was filled and capped. Within 10 minutes after shedding, the blood from this syringe was introduced under mineral oil into 4 ml. Pyrex test tubes through a long needle. Ten minutes after venipuncture the blood from one of these small tubes was withdrawn from under the oil into a clean 5 ml. syringe and was immediately introduced into the diffusion dishes for analysis with the control sample. This step was introduced to make sure the additional handling and storage under oil did not alter the ammonia concentration.

One-half the remaining oil-sealed tubes were quick-frozen in a solid CO_2 (dry ice) and alcohol bath; the other one-half were allowed to stand at room temperature. At 1, 2, 3, 4, 6, and 24 hours after shedding, one of the samples kept at room temperature and one frozen blood sample were analyzed as a pair.

Once the samples were quick-frozen, they were held in the frozen state in a standard electric freezer. The frozen samples were quickly thawed prior to analysis by rolling them gently between the palms of the hands for about one minute.

Once again, blood from a patient known to have an elevated blood ammonia level was sampled in the same manner. To assess the effect of cooling short of freezing point, blood from an additional subject was sampled in the same way; ice and water was used in place of the solid CO_2 and alcohol bath.

RESULTS

The data from Group I are shown in Fig. 1. They clearly show no change in ammonia concentration during the first minutes after sampling, whether the samples fall in the normal range of, or have elevated, blood ammonia levels.

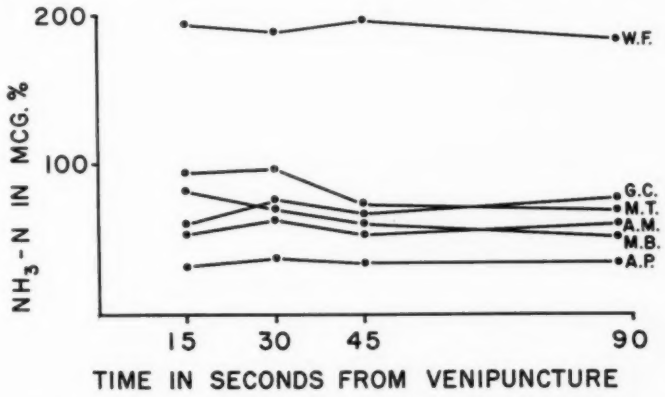

Fig. 1.—Blood NH_3-N concentrations during the first 90 seconds after venipuncture.

The data for the preservation of blood for ammonia determination are presented in Table I and Fig. 2. Significant increase in the production of blood ammonia in samples at room temperature is seen to begin after 2 hours, and to continue steadily thereafter. The frozen samples, however, showed almost no change in value. As can be seen, the average increase in ammonia concentration in the frozen samples after 24 hours was only 12 meg. per 100 ml. (meg. per cent), and in no sample did the increase exceed 15 meg. per cent or the level go beyond the normal range. Attention is drawn to the fact that one pair

TABLE I. BLOOD NH_3-N CONCENTRATIONS (MCG. %) IN TEN NORMAL SUBJECTS AT VARIOUS TIME INTERVALS AFTER VENIPUNCTURE WITH PRESERVATION BY FREEZING

SUBJECT	TIME AFTER VENIPUNCTURE (HRS.)													
	10 MIN.		1		2		3		4		6		24	
	CONTROL	RM. T.	FRZ.	RM. T.	FRZ.	RM. T.	FRZ.	RM. T.	FRZ.	RM. T.	FRZ.	RM. T.	FRZ.	RM. T.
S. C.	37	43	67	66	43	62	68	88	70	92	93	127	83	340
T. A.	88	72	75	67	61	70	55	92	85	129	78	166	49	545
L. E.	94	68	51	63	74	73	57	77	73	72	55	96	116	282
S. T.	69	34	63	44	87	73	57	87	57	88	71	137	72	361
G. A.	54	61	68	54	55	56	51	116	100	112	62	123	63	333
B. R.	31	71	103	72	59	47	37	66	97	107	71	133	51	280
O. S.	75	70	48	63	54	57	46	76	58	130	75	141	73	316
P. V.	51	39	51	41	43	56	74	108	149*	248*	64	156	64	361
K. O.	39	23	96	97	58	80	55	98	80	118	87	140	97	382
I. R.	55	42	48	55	43	75	40	63	54	86	44	106	45	507
Mean	59	52	67	62	58	65	54	87	74	103	70	133	71	371
Standard Deviation											15	21	20	89

*See text.

of 4 hour samples (P. V.) was omitted from the calculation of mean values because they disagreed considerably not only with the other 4 hour samples, but also with the remaining samples from the same subject. Since both values were unexpectedly high and differed by approximately the same amount from

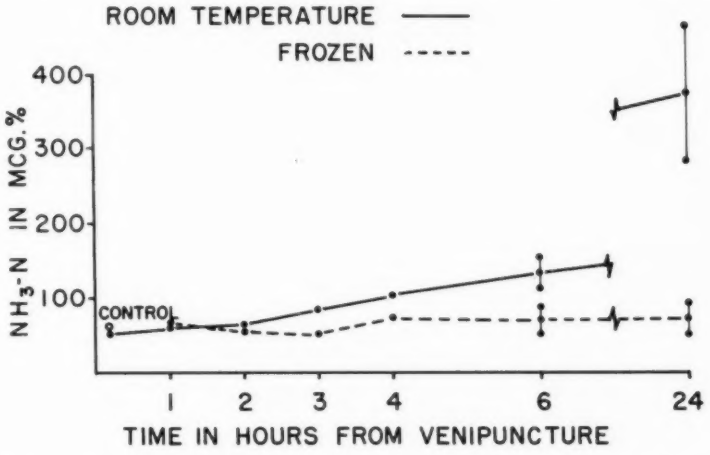

Fig. 2.—Mean blood NH_3-N concentration in 10 normal subjects at various time intervals after venipuncture showing the effect of quick freezing. (Plus and minus one standard deviation is shown by the vertical bars.)

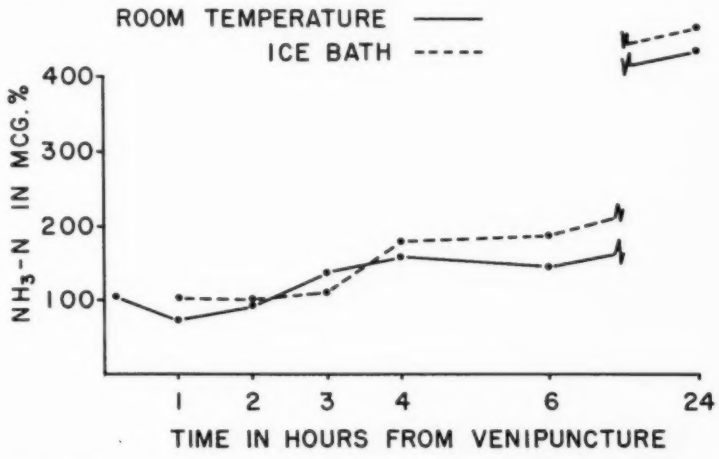

Fig. 3.—Blood NH_3-N concentrations at various time intervals after venipuncture showing the effect of cooling in an ice bath.

their predicted levels, the aberrent results were felt to be due to an unknown technical error, perhaps contamination. Blood from a patient with hepatic coma (W. F.) was also studied. The control level was 194 mcg. per cent and, 24 hours later, the frozen sample was relatively unchanged at 207 mcg. per cent.

Fig. 3 shows the effect of ice-bath cooling upon a blood sample. Again a significant increase in blood ammonia which was like the increase found when the sample was held at room temperature was noted after 2 hours.

DISCUSSION

The concept that there is no circulating free ammonium ion and that its concentration increases to the usually measured values during the first few minutes after blood is shed was advanced by Conway[2] and later supported by White and co-workers.[6] Our results and those of other workers[7, 8, 10, 11] fail to show any increase in blood ammonia concentration during the first few minutes. The difference is found in the handling techniques. Conway and White both used pipettes to transfer the shed blood; the others used anaerobic technique with sealed syringes. Jacquez and associates[11] recently emphasized the fact that if CO_2 escapes from the sample the pH will rise causing (1) a change in the distribution of intra- and extracellular ammonium ion, and (2) increased evolution of ammonium ion by enzymatic protein breakdown. Thus, anaerobic handling is necessary.

In order to prevent the rise in blood ammonia which starts 15 to 20 minutes after venipuncture even in sealed containers, several techniques have been tried. Dimond[12] showed the inhibiting effect of heparin upon adenylic deaminase, and its use as the anticoagulant of choice has been recently emphasized by Jacquez and associates[11] and by deGroote and Vandenbroucke.[14] Magid and collaborators[13] found that an occasional sample of heparin can contain as much as 5,560 meg. per cent and thus can be a source of significant error. However, the usual concentration of ammonia in heparin is 300 meg. per cent or below, and the amount used to coat a 5 ml. syringe (0.1 ml., calculated by differential weighing) can cause at most a 10 per cent error in the final result where the ammonia level is in the normal range. The error would be less (in percentage terms) in samples with elevated levels.

When the blood sample is cooled in an ice bath an additional 2 hours is allowed before the test must be performed. Preparation of plasma[11] or a protein-free filtrate[10] prior to icing is time consuming in itself but gives more consistent results when analysis is delayed up to 2 hours. Still this does not allow storing of samples to be analyzed at a central laboratory or at a certain time of day.

Quick-freezing of blood samples for ammonia determinations as described here prevents the usual rise in ammonia concentration for at least 24 hours. The disruption of the cell membrane by freezing obviously introduces an error in measurement of circulating blood ammonia, but that error is evidently insignificant. Freezing will allow greater laboratory economy, and make the test available to those previously denied it by factors of time and distance. In the light of recent work,[10, 11] it is possible that still greater consistency and reliability would be obtained by using frozen plasma or a frozen protein-free filtrate if the sample were to be held for more than 24 hours. It is also likely that with the advent of freezing as a method of preservation of whole blood for transfusion, the high content of ammonia in stored bank blood[9] will no longer be a clinical problem.

SUMMARY

1. A method is presented for the preservation of whole blood for ammonia analysis by quick freezing.

2. The ammonia concentration of the quick-frozen sample does not vary significantly up to 24 hours after the blood has been shed.

3. No significant increase in blood ammonia concentration was found from 15 seconds to 15 minutes after venipuncture when anaerobic technique was used.

The technical assistance of Mrs. Ann Powell is most gratefully acknowledged.

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THE DETERMINATION OF ADRENAL CORTICAL α-KETOLIC STEROIDS BY QUANTITATIVE PAPER CHROMATOGRAPHY

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TOUCHSTONE and Hsu[1] and others[2] have reported quantitative procedures for the determination of α-ketolic steroids in biologic fluids. These methods involved extraction of the fluids and paper chromatographic separation of the steroid components present in the extracts. The chromatograms were visualized by spraying them with alkaline blue tetrazolium.* The resultant formazans were eluted and determined colorimetrically.

We have developed a modification of this technique which permits direct densitometric determination of α-ketolic steroids on the original chromatogram. This method has been routinely applied to the identification and quantitative determination of adrenal corticoids secreted by rat adrenal tissue in vitro.[3] It is probable that the procedure can be further modified to permit the determination of these steroids in other biologic fluids.

MATERIALS AND METHODS

Reagents.—Toluene (analyzed reagent), methanol (analyzed reagent), formamide (analyzed reagent), glacial acetic acid (analyzed reagent), sodium hydroxide (analyzed reagent), blue tetrazolium,† and dichloromethane.‡

Apparatus.—Cylindrical glass chromatographic jars 6 by 18 inches.§

Special Instrumentation.—Model R.B. Analytrol, fitted with a 550 mμ interference filter and B-2 light balancing cam.‖ Paper kymograph strips 6½ by 21 inches, ruled in 1 mm. squares.¶

Paper.—Whatman No. 3 MM strips, 3 by 46 cm. Whatman No. 1 sheets, 17 by 21 inches.

Preliminary Chromatographic Preparations.—The inside of the chromatographic jars were lined with a sheet of Whatman No. 1 paper. The paper liner was then saturated with the lower phase of a solvent mixture prepared by shaking 400 ml. of toluene, 280 ml. of methanol, and 120 ml. of water in a separatory funnel. Enough of both phases was then poured into the bottom of the jar to insure contact of the solvent with the bottom edge of the paper liner. The jars were kept in a constant temperature cabinet regulated to 25 ± 1° C.**

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*3, 3′-(3, 3′-dimethoxy-4, 4′ biphenylene)-bis (2, 5-diphenyl-2H-tetrazolium chloride).
†Matheson Coleman and Bell.
‡Eastman Organic Chemicals, No. 342.
§Harshaw Scientific, Type H-10984.
‖Beckman, Spinco Division.
¶Aloe Scientific, Type 68102.
**A constant temperature cabinet is not necessary if the room is maintained at a reasonably constant temperature.

The Whatman No. 3 MM strips were marked with a pencil across the width of the paper at a distance of 10 cm. from the top edge. A circle 1.5 cm. in diameter was drawn with a pencil at the center of the line. The circles were used as guides so that the material to be separated was distributed in spots of the same diameter in all chromatograms.

Chromatographic Procedure.—The dichloromethane extracts of the adrenal incubates (usually 8 to 10 ml.) were evaporated to dryness in 15 ml. conical centrifuge tubes at 37° C. under a stream of N_2. The residues were reconstituted in a smaller volume of solvent by allowing heated dichloromethane vapor to condense and run down the sides of the tubes until about 0.5 ml. of liquid had accumulated. The solvent was again evaporated to dryness. Just prior to application to the paper strips, the residues were reconstituted to about 0.2 ml. in the same way.

The concentrated extracts were applied to the chromatograms in 20 μl portions with a micropipette. The solvent was allowed to evaporate at room temperature in still air. The sizes of the spots were carefully controlled. The area of each spot corresponded to the area within the pencilled circle at the starting line.

The chromatograms used for the preparation of the standard curves were made in a similar way. Solutions of the reference steroids* were prepared in dichloromethane. Aliquots equivalent to from 2 to 20 mcg. were evaporated within the circle at the starting line; the same precautions described previously were used.

Two chromatograms were placed in each jar and allowed to equilibrate overnight. The next morning the solvent troughs were filled with the upper phase of the toluene-methanol-water solvent. The chromatographs were allowed to develop by descending chromatography for 105 minutes. During this time the solvent front had moved to within 3 inches of the bottom of the strip. The developed strips were then air dried at room temperature.†

The paper strips were then scanned in the Analytrol. This blank recording was made because background optical density fluctuates considerably because of variations in paper structure. The blank tracings were used as a base line in the interpretation of the recordings obtained after blue tetrazolium visualization. The procedure used for scanning the chromatograms will be described later.

Visualization of the Chromatograms.—The alkaline blue tetrazolium solution was prepared just before use. Five milliliters of a formamide solution containing 5 mg. blue tetrazolium per milliliter was stirred into 500 ml. 2N NaOH. The resultant solution, adjusted to 25 ± 1° C., was poured into a clean 12 by 18 inch shallow enameled tray. The chromatograms were carefully lowered onto the surface of the liquid. Extreme care must be taken not to damage or distort the paper since it is greatly weakened by exposure to the alkaline reagent. The chromatograms were immersed in the liquid by gently rocking the tray. After 5 minutes the developing solution was carefully decanted away from the strips. The chromatograms were then washed in the tray with 500 ml. of 2 per cent acetic acid. Neutralization was hastened by gently rocking the tray. The chromatograms were left in contact with the solution for 5 minutes. The acid was decanted and the chromatograms were given a final wash with water. The strips were then removed from the tray and were carefully blotted free of excess moisture with paper towels. Finally, the strips were air dried at room temperature. The strips were scanned within 3 hours after development since the formazan pigment slowly becomes decolorized on standing.

Scanning Procedure.—The scanning operation was carried out with the Analytrol recording and integrating densitometer. The instrument was equipped with 550 mμ interference filters and the B-2 light balancing cam. A slit width of 4 mm. was used.

*Aldosterone was obtained from the Endocrinology Study Section, National Institutes of Health; the dehydrocorticosterone was obtained from Dr. E. Albert of Merck Sharpe & Dohme (Div. of Merck & Co., Inc.); and Dr. O. V. Dominguez, Department of Biochemistry, University of Utah College of Medicine, supplied the 6β-hydroxydesoxycorticosterone.

†The chromatographic procedure is essentially that described by Bush.[5]

The procedure used for scanning the chromatograms was essentially that described in the Analytrol instruction manual. The zero and calibration set point adjustments were made with the area of the chromatogram which had the lowest light absorption in front of the slit. The zero point for the instrument was set with the pen 4.0 cm. from the bottom edge of the paper. The calibration set point was determined by adding 4.0 cm. to the value obtained from the calibration set point table in the instruction manual. After these adjustments were made the strip was moved until the starting line was slightly to the right of the slit. The scanning operation was then carried out in the usual manner.

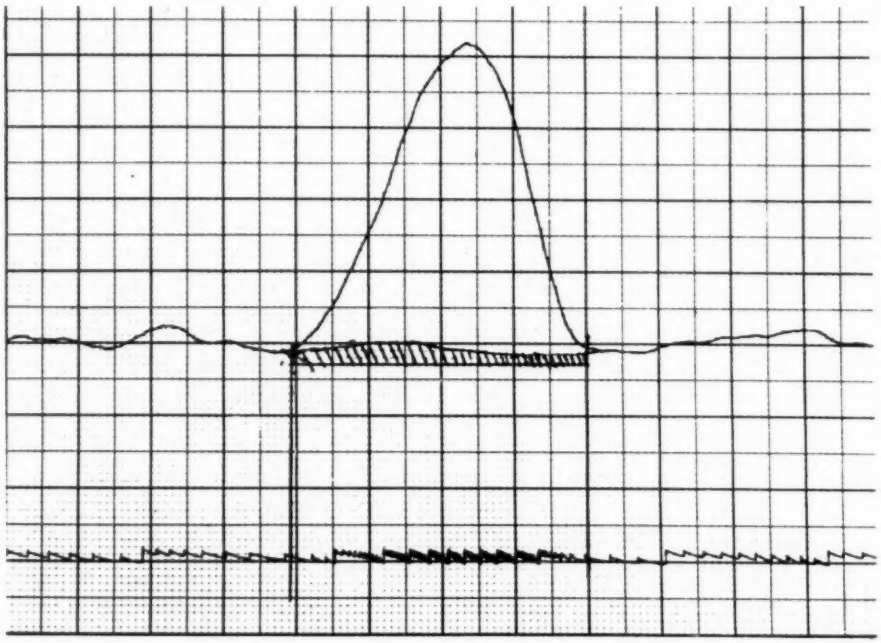

Fig. 1.—A representative peak given by 15 mcg. of cortisone. A total of 99.8 integration marks appear between the vertical lines. The cross-hatched area between the zero line and the background line is equivalent to 9.3 integration marks. The corrected area is equal to 90.5 integration marks.

Calculations.—The experimental recording was superimposed on the blank over the glass of an illuminated tracing box. The base line obtained before blue tetrazolium visualization was then traced on the final recording. Care was taken to obtain the best possible match of the 2 traces in the steroid free areas of the chromatogram. The areas bearing significant amounts of formazan were clearly delineated at the points at which the 2 curves separated. These areas were ruled off with vertical lines intersecting the integration trace. Horizontal lines were drawn underneath the peaks at the zero line 4.0 cm. from the bottom of the chart. The total integration marks were counted between the vertical lines and corrected for the area between the background line and the zero line (Fig. 1). The corrected count was found to be adequately reproducible, and was characteristic of the quantity of the steroid present on the chromatogram. In practice, unknown quantities of steroid were calculated from standard curves prepared from chromatograms bearing known amounts of the reference steroid.

RESULTS

Representative standard curves of several adrenal corticoids are shown in Fig. 2, *A*, *B*, *C*, and *D*. Each point represents a single determination. The

TABLE I. REPRODUCIBILITY OF STANDARD CURVE

ADRENAL CORTICOID (MCG.)	TOTAL INTEGRATION MARKS 1	2	3	4	AVERAGE ± S.D.
Cortisone.—					
2	18.3	20.5	17.5	20.6	19.2 ± 1.6
4	26.7	28.2	31.6	29.7	29.0 ± 2.1
6	35.2	42.0	37.1	32.4	36.7 ± 4.1
10	54.6	58.6	59.0	62.3	58.6 ± 3.2
Corticosterone.—					
2	23.1	20.2	24.2	21.7	22.5 ± 1.7
4	43.9	37.5	43.0	37.2	40.4 ± 3.5
6	57.9	52.1	60.9	57.8	57.2 ± 3.7
10	76.5	81.5	87.0	77.7	80.7 ± 4.7
Desoxycorticosterone.—					
2	19.0	22.0	21.6	22.0	21.2 ± 1.4
4	39.6	37.9	39.4	34.0	37.7 ± 2.4
6	57.4	45.1	45.7	47.6	49.0 ± 6.7
10	75.8	71.4	73.7	74.2	73.4 ± 1.9

curves are nearly linear in the range from 2 to 10 meg. The curves shown represent the line of regression determined by least square analyses. It was apparent that the curves were not truly linear even in this range. The data in Fig. 3 demonstrate that useful nonlinear curves were obtained with corticosterone in the range of 2 to 25 meg. Similar results were obtained with desoxycorticosterone and cortisone. The curves were adequately reproducible as shown by the data in Table I. The individual values in the table are the

TABLE II. PRINCIPLE α-KETOLIC STEROIDS SECRETED IN VIVO BY SPRAGUE-DAWLEY RAT ADRENALS*

INCUBATION NO.	AVERAGE BODY WEIGHT (GM.)	ADRENAL TISSUE WEIGHT (MG.)	μG. STEROID/HR./100 MG. TISSUE ALDOSTERONE†	CORTICOSTERONE	DEHYDROCORTICOSTERONE
Females.—					
1	130	128	1.0	7.5	1.5
2	132	138	1.5	8.1	1.1
3	138	140	1.6	10.7	2.1
Average	133	135	1.4	8.8	1.6
Males.—					
4	158	126	2.9	8.7	0.8
5	137	107	2.8	9.5	0.8
6	155	124	2.0	10.1	1.0
Average	150	119	2.6	9.4	0.9

The combined halved adrenals of 4 animals were used in each incubation. 100 mu. of ACTH was added to the incubation fluid for every 100 mg. of adrenal tissue.

*Traces of other α-ketolic steroids were present.

†Aldosterone levels computed as cortisone.

result of 4 independent determinations. The standard deviations from the means are a measure of the reproducibility of the entire chromatographic procedure. From these and similar data it is apparent that the standard error of a single determination was not more than 20 per cent of the observed value and averaged 10 per cent throughout the 2 to 10 mcg. range. In routine use a set of standard chromatograms was run with the experimental chromatograms. Similar experiments were carried out using Whatman No. 1 paper strips. Whatman No. 3 MM consistently gave significantly better results.

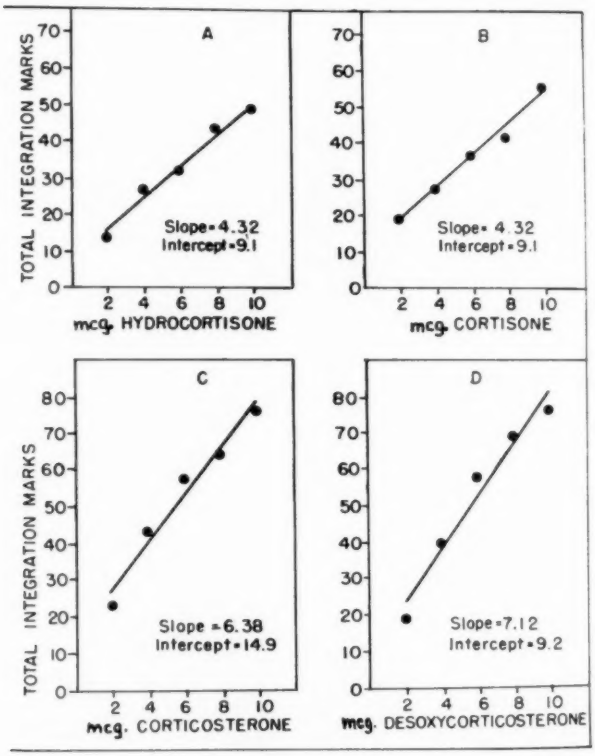

Fig. 2.—Typical lines of regression for 4 common α-ketolic steroids calculated by least square analysis. A linear relation was assumed between the quantity of the steroid placed on the chromatograms and the area under the peaks of the Analytrol recording of the developed chromatogram. Each point represents a single value from the same set of chromatograms processed simultaneously. Similar results were obtained with 6β-hydroxydesoxycorticosterone and dehydrocorticosterone.

Several recovery experiments were carried out in which known amounts of corticoids were added to 10 ml. of dichloromethane. The solutions were then concentrated and transferred to paper strips using the procedures previously described. The recoveries averaged from 88 to 102 per cent.

Table II shows the results of the application of these techniques to the analysis of the α-ketolic steroids secreted in vitro by adrenal halves of untreated young male and female Sprague-Dawley rats.[4] The combined tissue was pre-incubated in 5.0 ml. of Krebs Ringer bicarbonate glucose medium for ½ hour at

37° C. with a gas phase of 95 per cent O_2 and 5 per cent CO_2 bubbled through the medium. The incubational media were then replaced with equal volumes of the same medium containing, in addition, 100 mu. of Acton "X" ACTH* per 100 mg. of adrenal tissue. The second incubation was carried out for 1 hour

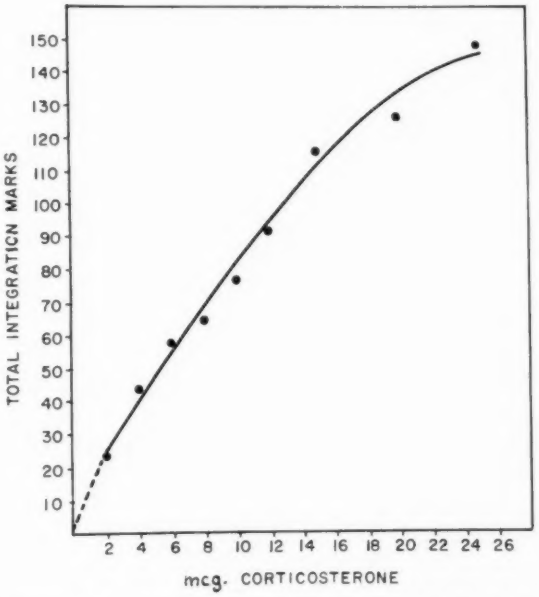

Fig. 3.—An extended representation of the same curve shown in Fig. 2, *C*. Note deviation from linear behavior over the greater range. All points represent single values from a single set of chromatograms run simultaneously.

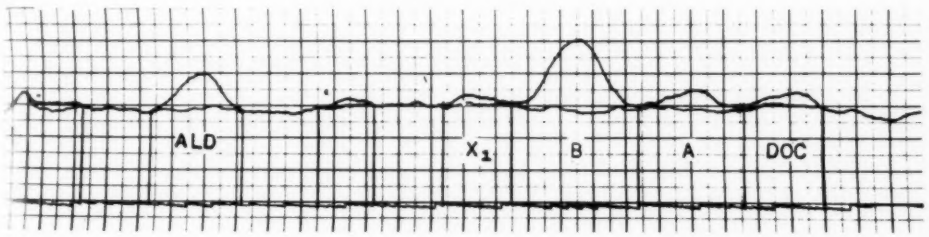

Fig. 4.—The peak labelled *ALD* had the chromatographic mobility of authentic aldosterone [o]n the chromatogram was made in solvent systems which gave good separation of hydro[c]ortisone and cortisone. The peak labelled X_1 has been tentatively identified as 6β-hydroxy[d]eoxycorticosterone. The peaks labelled *B*, *A*, and *DOC* are corticosterone, dehydrocorti[c]osterone, and desoxycorticosterone, respectively.

[u]nder the same conditions. The incubational media were decanted from the [t]issue and extracted with dichloromethane. The extracts were processed and [a]nalyzed by the procedures given previously. The aldosterone secretion was [c]alculated using a cortisone standard curve.

*We are indebted to Mr. K. Antoft of Nordic Biochemicals for the supply of Acton "X" [A]CTH.

Fig. 4 shows a recording made from a chromatogram of the steroids secreted in vitro by the adrenals of female Sprague-Dawley discard breeder rats. The adrenals were obtained from animals which had arteriosclerotic lesions which were grossly visible.[3]

DISCUSSION

The method produces permanent records which permit a rapid and convenient comparison of the effect of experimental conditions on the spectrum of α-ketolic steroids present in in vitro incubates of adrenal glands. The Rf values were found to be highly reproducible when the chromatograms were properly equilibrated and not overloaded. The techniques could presumably be adapted to other biologic fluids.

SUMMARY

A quantitative method for the direct determination of α-ketolic steroids on paper chromatograms is described. The chromatograms are scanned with a recording and integrating densitometer after blue tetrazolium visualization. Hydrocortisone, cortisone, corticosterone, aldosterone, and other adrenal corticoids present in extracts of biologic fluids can be rapidly and conveniently determined. The method has been applied to the determination of the adrenal corticoids secreted in vitro by the rat adrenal gland.

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A PRACTICAL TEMPERATURE-COMPENSATED HAND REFRACTOMETER (THE TS METER): ITS CLINICAL USE AND APPLICATION IN ESTIMATION OF TOTAL SERUM PROTEINS

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REFRACTOMETRY has been applied to the determination of total serum solids,[1] and total serum proteins.[2-4] The validity of this method of total solids determination has been demonstrated repeatedly, but its accuracy in the estimation of serum proteins in specimens with varying albumin and globulin content and ratios has been questioned. Rubini and Wolf[1] demonstrated the accuracy of refractometric determination of total solids in serum with a protein content range of 1.77 to 13.9 Gm. per 100 ml. and albumin to globulin ratio of 0.13 to 14. In their study, no attempt was made to estimate total serum protein from total serum solids as measured by the refractometer.

Refractometry has not received wide experimental or clinical use because the method has been tedious and technically difficult. Wolf[5] noted the development of a temperature-compensated hand refractometer* for sugar analysis in the canning industry. An instrument was made (Fig. 1)† in which total solids of serum and urinary specific gravity could be measured directly and read on a scale (Figs. 2 and 3) incorporated in the instrument.

The present clinical study was designed to answer the following questions:

1. Is the instrument suitable for routine clinical and research use in terms of durability, simplicity of the method, time consumed in running several specimens, and reproducibility of results in the determination of total serum solids?

2. Can a clinically reliable estimation of total serum proteins in postsorptive specimens be made from the refractometric determination of total solids despite wide variation in total solids, albumin and globulin content, and ratio?

METHODS

Seventy-seven postabsorptive specimens were examined. One drop of serum was placed in the refractometer chamber (Fig. 4) and the grams of total serum solids per 100

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†In collaboration with the American Optical Company.

Gm. serum was read directly on the scale. Readings on each specimen were made independently by 2 observers and many sera were re-examined up to 14 days after the initial reading. Serum osmolality determined by the freezing point method, utilizing a Fiske Osmometer, varied from 282 to 310 mOsm per liter. Total protein, albumin, and globulin were determined by the biuret method[6]; the ranges were 4.9 to 8.7 Gm. per cent, 3.2 to 5.2 Gm. per

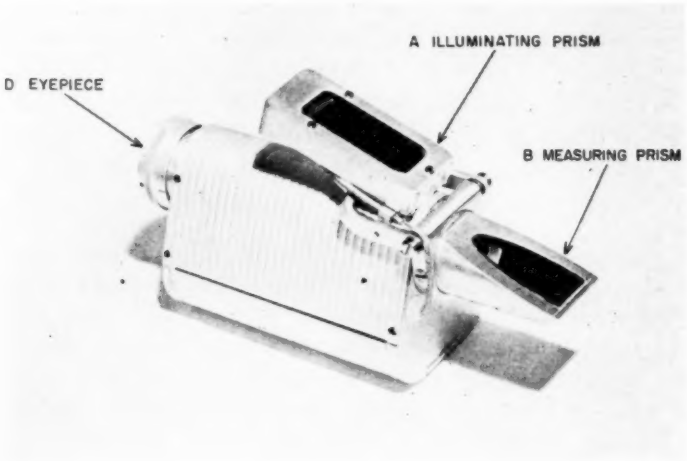

Fig. 1.

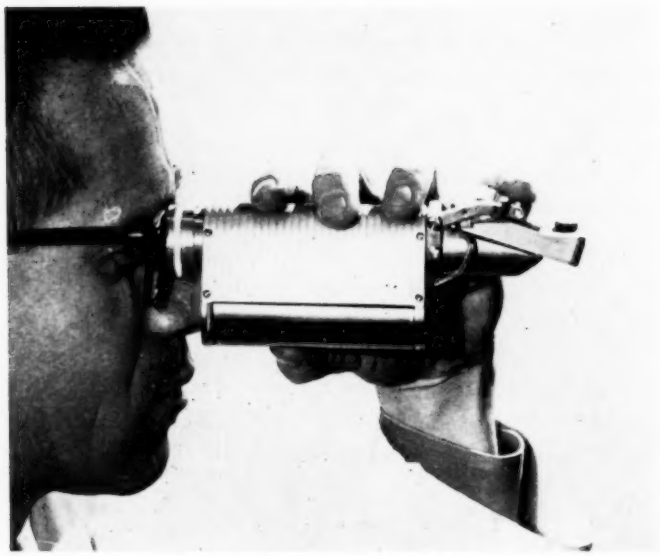

Fig. 2.

cent, and 1.7 to 5.4 Gm. per cent, respectively. The range of albumin to globulin ratios was 0.59 to 2.31. Routine methods[7] were used for the determination of total serum cholesterol with a range of from 163 to 331 mg. per cent. No specimens were grossly lipemic, but several were grossly icteric.

Statistical methods included correlation techniques and the determination of measures of central tendency, i.e., the median, the harmonic mean, and measures of dispersion.[8]

RESULTS

The questions posed in the experimental design may be answered in the affirmative.

1. The TS meter proved to be durable in withstanding the usual trials and tribulations of a frequently used and occasionally abused laboratory tool. The methodology and technique were simple. During use it was necessary to wipe the serum from the capillary chamber with absorbent tissue before it was refilled for the next determination. Since serum is rather adhesive, the chamber was cleaned with distilled water after each group of 5 specimens was run.

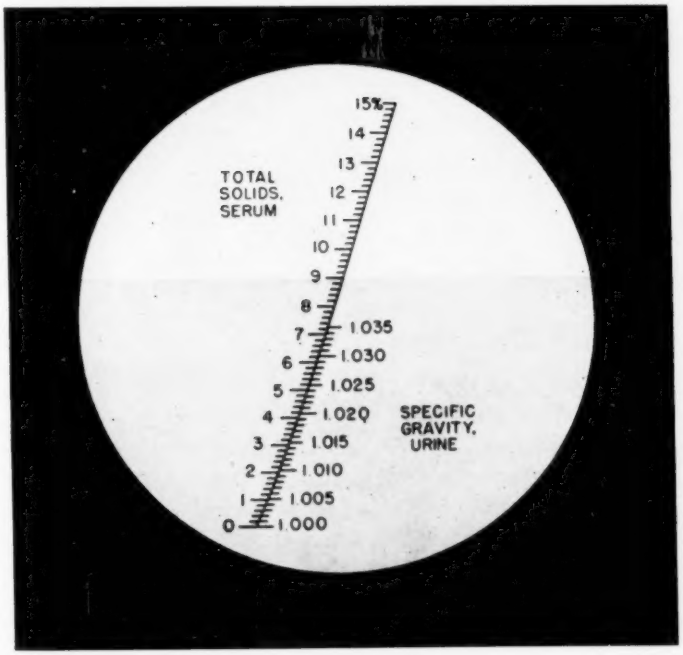

Fig. 3.

The technique allowed 20 determinations in 30 minutes working at a leisurely pace. The results were reproducible, not only on the same day with different observers but also on re-examination up to 2 weeks after the initial determination. The total performance of the instrument in terms of easy, practical day-to-day use was excellent.

2. A reliable estimate of total serum proteins could be made from the refractometric reading of the total serum solids. Fig. 5 is a scattergram of the specimen values with the regression (least squares) line superimposed. The 2 variables (X and Y) of the regression equation ($\underline{Y} = a + bX$) were

not mutually independent. However, the stability of the proportions was established by computing the harmonic mean ratio of total serum proteins to total serum solids and evaluating the significance of the deviations from the regression (least squares) line. The differences were not proved to be statistically significant.

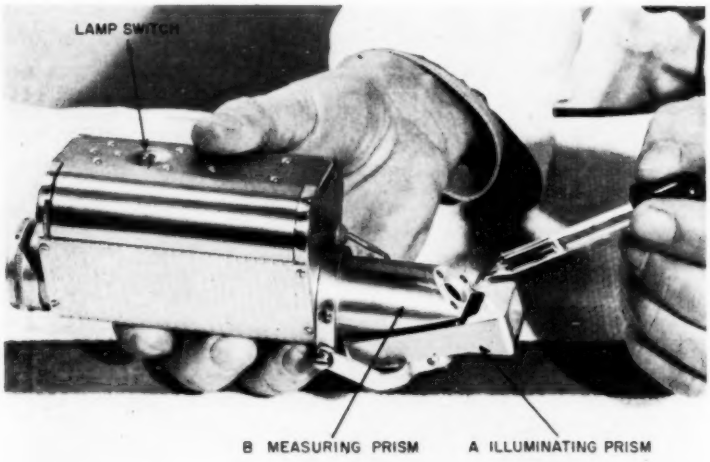

Fig. 4.

The standard error of estimate ($\bar{S}_y$) has been determined to be 0.15 and 95 per cent of all $\underline{Y}$ values (total serum proteins estimated), for specimens such as these can be expected to fall within the range of $a + bX \pm 1.96\ \bar{S}_y$ where the estimate is based on the refractometric evaluation of total serum solids.

TABLE I. ESTIMATED VALUES OF TOTAL PROTEIN FOR VALUES OF TOTAL SOLIDS AS DETERMINED FOR POSTABSORPTIVE SERA BY THE REFRACTOMETRIC METHOD

INTEGRAL PORTION OF TOTAL SOLIDS	DECIMAL PORTION OF TOTAL SOLIDS									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
5.0	3.70	3.79	3.88	3.97	4.06	4.14	4.23	4.32	4.41	4.50
6.0	4.59	4.68	4.77	4.86	4.95	5.04	5.12	5.21	5.30	5.39
7.0	5.48	5.57	5.66	5.75	5.84	5.93	6.02	6.10	6.19	6.28
8.0	6.37	6.46	6.55	6.64	6.73	6.82	6.91	7.00	7.08	7.17
9.0	7.26	7.35	7.44	7.53	7.62	7.71	7.80	7.89	7.97	8.06
10.0	8.15	8.24	8.33	8.42	8.51	8.60	8.69	8.78	8.87	8.95
11.0	9.04	9.13	9.22	9.31	9.40	9.49	9.58	9.67	9.76	9.85
12.0	9.93	10.02	10.11	10.20	10.29	10.38	10.47	10.56	10.65	10.74

Conversion table total solids grams per 100 Gm. serum to total serum protein grams per cent. Example: total solids are determined to be 12.7; find 12.0 in the table stub and move across the table on line 12.0 to the column headed 0.7—the estimated value for total protein is 10.56 ± 0.15 Gm. per cent.

Values given in the body of the table represent the estimates of total protein contained in the total solids which are represented by the stub and column head values. The total protein values are computed by the equation: $\bar{Y}_e = 0.89 \times -0.75$ where x = (stub value plus head value) and Y_e = the best estimate of total protein for a given x.

For this sample, the means are $\bar{X} = 9.06$ SD ± 0.70, $\bar{Y} = 7.32$ SD ± 0.59, the regression coefficient b = +0.89, the regression constant a = −0.75, the coefficient of correlation r = +0.94.

Table I provides a ready conversion reference for use in the estimation of the total serum proteins from the refractometric value of total serum solids.

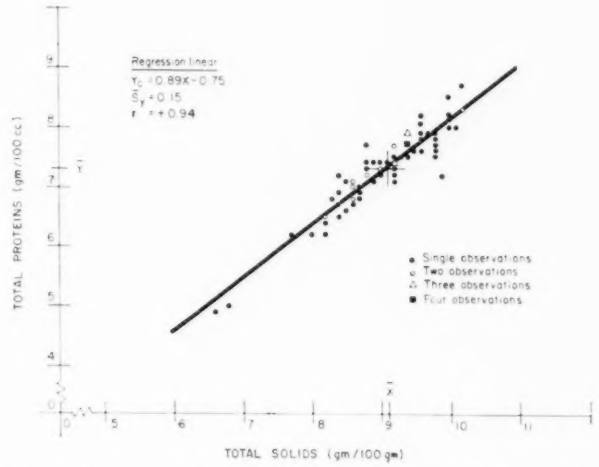

Fig. 5.—Total protein in postabsorptive serum as determined from total serum solids.

DISCUSSION

A practical instrument which requires only 1 drop of fluid to determine rapidly the total solid content has important application in clinical and research medicine. In clinical states of disturbed hydration, such as acute dehydration of burns, the ability to determine rapidly from a drop of sera the total serum solids (and water) allows proper fluid therapy from hour to hour. In the differentiation of oliguria of acute renal failure from that of dehydration it offers an additional guide. The research scientist working with small animals is often limited by the quantity of body fluids available for analysis. The 1 drop of fluid required by the TS meter for total solids assists in this common problem.

A reliable estimation of total serum proteins may be made from the refractometric determination of total serum solids in the postabsorptive state. Grossly lipemic serum was not studied, but icteric serum did not prevent a satisfactory reading nor bias the data. The method warrants trial as a screening procedure when an alteration of total serum protein is suspect and in following the course of total serum proteins when an alteration is found.

SUMMARY

The TS meter, a temperature compensated hand refractometer of recent development for determination of total solids of human sera and other body

fluids, has been tested for over-all laboratory performance. The test specimens were human serum. The instrument proved highly satisfactory in terms of reproducibility of results, economy of time, and simplicity of technique. Only 1 drop of test serum was required.

A correlation was made between the refractometer reading for total serum solids and total serum protein. A linear regression line was constructed with an r value of 0.94 and an $\bar{S}_y$ of 0.15. A conversion table was computed from the formula $Y = a + bX$. A statistically reliable estimation of total serum proteins may be made from the refractometric determination of total serum solids.

The value of the instrument in clinical and research medicine is noted.

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A RAPID METHOD FOR THE PREPARATION OF BACTERIOPHAGE SUSPENSIONS FOR STAPHYLOCOCCAL TYPING

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THE method commonly in use for the production of high titer phage suspensions for bacteriophage typing of staphylococci is the soft agar layer technique described by Swanstrom and Adams.[1] In this method the propagating host strain and phage suspensions are mixed in 0.7 per cent agar and poured over a hardened 1.5 per cent agar plate. After incubation for 18 to 24 hours the soft agar layer is scraped off and centrifuged, and the supernatant is filtered. The filtrate is assayed, diluted, and used for typing.

A method which is less tedious than the soft agar layer method has proved to be highly satisfactory in our laboratory. This method is a modification of the technique suggested by Mandle,[2] and White, Foster, and Knight.[3]

MATERIALS AND METHODS

The original bacteriophage suspensions and propagating bacterial strains were supplied by Dr. John E. Blair.* A series of 22 phages were used. These included phage Strains 3A, 3B, 3C, 6, 7, 29, 42D, 42E, 47, 52, 52a, 53, 54, 55, 71, 73, 75, 77, 79, 80, 81, and Va_4.

Cultures of the propagating strains were incubated in trypticase soy broth at 37° C. for 14 to 18 hours. A transfer of 0.1 ml. of this culture was made into 10 ml. of trypticase soy broth in a tube suitable for aeration (Fig. 1). Aeration was accomplished by a small Marco air pump similar to an aquarium pump. The pump was connected by rubber tubing to a manifold capable of providing aeration for a number of tubes simultaneously. The manifold consisted of a series of small screw valves connected by rubber tubing, and attached to a wooden strip mounted on a constant temperature water bath (Fig. 2).

The cultures were aerated until the optical density reading on a Coleman Junior spectrophotometer at 485 mμ was between 0.04 and 0.07. This represents approximately 10^8 organisms per milliliter. One milliliter of phage suspension (approximately 10^7 phage particles per milliliter) was added, and the culture was aerated for 5 hours or until clear. The tubes were centrifuged for 10 minutes at 3,000 r.p.m. and the supernatant was collected. In cases where centrifugation failed to produce a sterile supernatant the suspension was filtered through a Selas filter. Tenfold dilutions of the supernatant were made in broth and tested against the propagating strains. The procedure was repeated when necessary until the suspension, diluted to 10^{-5}, showed confluent lysis against the propagating strain.

RESULTS

Stock phage suspensions with a potency suitable for typing were obtained in all cases by aeration of the culture and addition of phage in proportion to

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the number of bacteria present. No attempt was made to produce titers higher than those necessary to produce confluent lysis at a dilution of 10^{-5}. A tenfold rise in titer was observed in 6 phage strains after the treatment as outlined. Cultures of 13 of the strains tested became clear.

Phage suspensions in which propagating Strains 47, 80, 73, 42D, and 42E were used failed to show a rise in titer comparable to that of the other strains. When the incubation time before addition of the phage was reduced to 0.5 hour, these strains also produced suitable titers of phage.

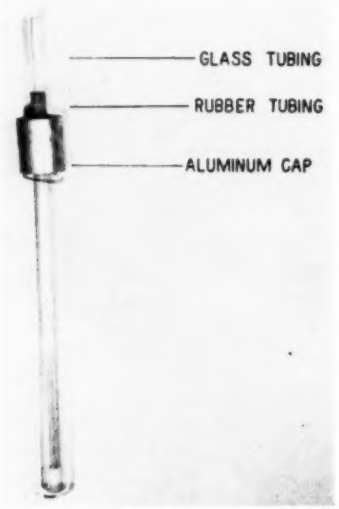

Fig. 1.—Aeration tube. The aeration tube consists of a standard 16 by 150 mm. tube fitted with an aluminum cap through which is passed a length of glass tubing sufficient to reach well into the medium. A short length of rubber tubing serves as a gasket between the glass tubing and the aluminum cap. The glass tubing is plugged with cotton.

DISCUSSION

The procedure described above for preparation of phage suspensions has been found to be much more satisfactory, in our laboratory, than the soft agar layer technique. Less time was involved in the production of high titer stocks of bacteriophage and no difficulty has been encountered by loss of potency of phage stock suspensions. The phage could be propagated, filtered, or centrifuged, and plates could be prepared for potency testing in an 8 hour period if desired. The use of undiluted phage stock for virus inoculum minimized the number of repetitions necessary to achieve satisfactory potency. Normally 3 or 4 repetitions were sufficient.

A lengthened latent period could explain the failure of Strains 47, 80, 73, 42D, and 42E to show a rise in titer comparable to that of the phage suspensions. Rountree[4] has reported the latent period during which new phage particles are produced within the cell to be between 40 and 50 minutes for typing phages. The phage strains that did not show the expected rise in titer are reported by

Rountree to have latent periods near the maximum of 50 minutes. In this extended latent period it would be possible for the number of bacterial cells to increase at a rate more rapid than that in which phage is reproduced within the cell. Shortening the incubation time before the addition of phage compensated for the slightly prolonged latent period and rises in titer were observed.

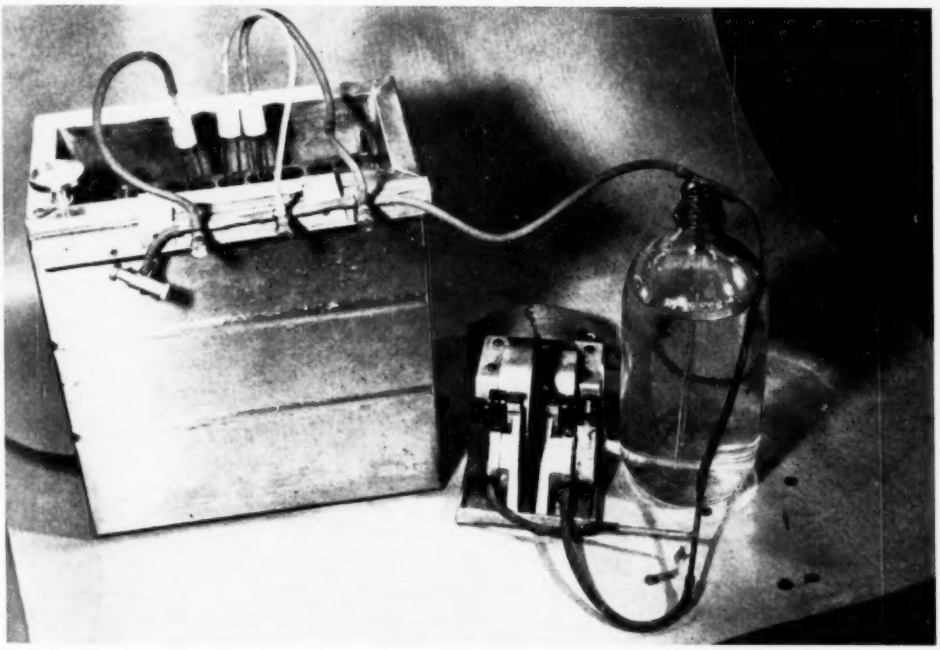

Fig. 2.—Arrangement for aeration of tubes. The manifold is fitted to a standard water bath. The water trap on the right allows release of excess air pressure.

SUMMARY

A rapid method for the preparation of high titer phage stocks has been described. This method utilizes rapid growth of the host under aeration and the addition of phage particles in approximately a 1:1 ratio to bacterial cells. Satisfactory results when 22 phage strains were used are reported.

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HYDROLYSIS OF CERTAIN PURE STEROIDAL CONJUGATES IN AQUEOUS SOLUTION

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MUCH has been written about the hydrolysis of steroidal conjugates in biologic fluids. Most reports rely upon the maximal yields of compounds recovered after a given method of hydrolysis. The efficiency of hydrolysis may also be determined by the administration of isotopically labeled steroids, the quantity of isotope which remains unextracted serving as an index of the degree.

Various highly purified steroidal conjugates have been donated to this laboratory by Schneider, who isolated them in the course of his excellent studies.[1] This made it possible to investigate various hydrolytic procedures in aqueous solutions and to determine the degree of hydrolysis based on the recovery of compounds as free steroids by extraction with polar solvents. These limited results are presented with the hope that they may provide useful information for other investigators.

MATERIAL AND METHODS

Steroidal conjugates were dissolved in 10.0 ml. water adjusted to a suitable pH in accordance with the method of hydrolysis employed as described in this article. The concentrations were equivalent to 60 to 100 mcg. of "free" steroids per sample. All tests were run in duplicate.

Following hydrolysis, the 17-ketosteroids were extracted 3 times with an equal volume of ethyl ether and were washed twice with 10.0 ml. of 10 per cent sodium hydroxide and twice with 10.0 ml. of water. The Zimmerman reaction was employed as modified in this laboratory,[2] for the measurement of released compounds.

Those experiments with the glucuronide of pregnane—3 α, 17 α, 21-triol-11, 20-dione (tetrahydro-E) were conducted by extraction with methylene chloride and measurement according to Silber and Porter.[3] Suitable aliquots of the original specimens were prepared for these determinations.

All results were corrected to account for differences in molecular weights of the free and conjugated steroids and colorimetric differences in reactions between various compounds according to Wilson.[4]

The hydrolytic procedures were conducted as follows. Acid: (1) continuous extraction with ethyl ether at pH approximately 0.9 for 48 hours according to Lieberman,[5] and (2) sulfuric acid, 12 per cent (v/v), added as a 40 per cent (v/v) solution and heated (80° C.) for 10 minutes.

Enzymatic preparations were used in varying concentrations according to the assays designated for the original package as supplied. (1) Bacterial β-glucuronidase* was used

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*Sigma Chemical Co., St. Louis, Mo.

at pH 6.5 in 0.1M phosphate buffer; (2) mammalian liver β-glucuronidase* at pH 4.5 in 0.1M acetate buffer; and (3) helix enzyme† containing both β-glucuronidase and sulfatase, in 0.1M acetate buffer at pH 5.2. Incubations were conducted at 37° C. for 24 or 48 hours. In addition, steroidal conjugates were incubated in the various buffer solutions without added enzyme. However, since not more than 2 per cent of any conjugate was so released within the time period employed, these results are not described. It should be noted however, that spontaneous hydrolysis of conjugates at neutrality was observed after many days or weeks, particularly of sodium dehydroepiandrosterone sulfate.

The following steroidal conjugates were employed. (1) Sodium androsterone glucuronide (AG); (2) sodium etiocholanolone glucuronide (EG); (3) tetrahydro-E glucuronide (TEG); (4) sodium dehydroepiandrosterone sulfate (DS); (5) sodium androsterone sulfate (AS); and (6) potassium etiocholanolone sulfate (ES).

RESULTS

The results are described in Table I as the percentage steroid extracted after each method of hydrolysis compared to the amount originally added to the specimen. No studies were conducted to guarantee preservation of the original structure of the steroid.

TABLE I

ENZYME (OR OTHER)			TIME	STEROIDS EMPLOYED (% HYDROLYSIS)					
SOURCE	G* (U./10 ML.)	S† (U./10 ML.)	(HOURS)	TEG	AG	EG	DS	AS	ES
Bacterial	500		48		74	100	3		
	1,250		24	54	94	100	2		
	1,250		48	79	100		8		
	2,500		24	61					
	2,500		48	81	100				
Mammalian	2,500		48	61	78	100	3		
	5,000		24		71		8		
	5,000		48	63	79	100			
	10,000		48	65	88	100			
Helix	325	600	48	74			78		
	650	1,200	48	75	70		99	7	81
	1,300	2,400	48				97	17	91
	2,600	4,800	48	98			98		
Twelve per cent sulfuric acid, 80° C., 10 minutes				0.9	94	100	63	43	24
Continuous extraction, pH 0.9				0	28	67	90	100	100

*G = beta glucuronidase.
†S = sulfatase.

DISCUSSION

Various methods of hydrolysis of steroidal conjugates in blood and urine have been employed. Bacterial and mammalian β-glucuronidase have been widely used for the hydrolysis of glucuronides. Sulfates have been released with success by continuous extraction at low pH[4] and more recently by solvolysis.[7] Enzymatic means for the hydrolysis of sulfates have also been recently introduced, but have not enjoyed general application to date.

When expressed as Fishman units, bacterial β-glucuronidase is generally more effective for the hydrolysis of all steroids than the mammalian enzyme.

*Ketodase, Warner-Chilcott Laboratories, Morris Plains, N. J.
†Endo Labs, Inc., Richmond Hill, N. Y.

However, since these two preparations are, in all probability, not identical, this standard method of comparison with phenophthalein glucuronide as the substrate would not appear to be valid. Neither source appears to effect complete hydrolysis of tetrahydro-E glucuronide in water. From these limited data, it appears that the glucuronide of etiocholanolone is most readily hydrolyzed among the compounds tested.

Both the sulfates of dehydroepiandrosterone and etiocholanolone are released by the snail sulfatase, whereas androsterone sulfate is relatively resistant to this enzyme. Attempts to employ this sulfatase for the hydrolysis of any of these three steroids added to human urine or blood have been unsuccessful in this laboratory.

Continuous extraction of steroid sulfates at pH 0.9 with ether appears to release the sulfates of the three compounds tested, but the glucuronides are only partially hydrolyzed in this manner. Stronger acid and heat produced variable results, but as reported earlier the glucuronide of tetrahydro-E is not at all affected by this method.[6] The procedure of solvolysis described by Burstein and Lieberman[7] may prove to be the ideal means for the hydrolysis of steroidal sulfates, although further study of this technique is awaited.

SUMMARY

The availability of a few steroidal conjugates has made it possible to study their hydrolysis by various means. These limited preliminary data are submitted as a source of information for other workers in the field.

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THE BLOOD SERUM AZURE A LEVEL IN THE DETERMINATION OF GASTRIC ACIDITY BY TUBELESS GASTRIC ANALYSIS

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TUBELESS gastric analysis with the azure A resin compound† developed by Segal, Miller, and Plumb[1] provides a reliable technique for the estimation of gastric acidity. Since the interpretation of the state of gastric acidity with this technique depends upon the amount of the azure A dye excreted in the total 2 hour urine collection after the oral administration of the azure A resin compound, the results may become invalid in individuals with urinary retention or in whom the collection of the urine is unreliable. This investigation was undertaken to circumvent the collection of urine in the above type of individual.

To attain this end, it was necessary both to develop an accurate method for the measurement of the minute quantity of azure A in any single blood collection and to determine the most appropriate time to withdraw venous blood after the oral administration of the dye compound.

METHOD

The first method attempted consisted of the extraction of the dye from the blood serum with butyl alcohol after precipitation of the blood protein with trichloroacetic acid. This technique failed both because of the incomplete extraction of all the azure A dye and interference at 630 mμ by a yellow pigment.

The method finally developed is as follows:

The venous blood drawn is allowed to clot and is then centrifuged at 4,500 r.p.m. for 10 minutes. One milliliter aliquots of the clear serum are then put into 2 separate 15 ml. centrifuge tubes to each of which are added 4 ml. distilled water, 1 ml. of 0.5N HCl, and 1 drop of a solution containing 195 mg. of $CuSO_4 \cdot 5H_2O$ in 100 ml. of 18 per cent HCl (6M).‡ After gentle shaking, both test tubes are placed in a continuously stirred propylene glycol bath at 90 to 95° C. for 10 minutes, and are then cooled in a cold water bath (20 to 25° C.) for 5 minutes. The tubes are then removed from the water bath and inverted; a finger cot is used to remove the water vapor from the tube. The optical density is then read on the

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†Diagnex Blue, E. R. Squibb & Sons, New York, N. Y.

‡If crystallized copper sulfate is unavailable, 1 drop of a solution made by mixing 40 ml. water and 10 ml. Benedict's solution with 50 ml. concentrated hydrochloric acid may be used.

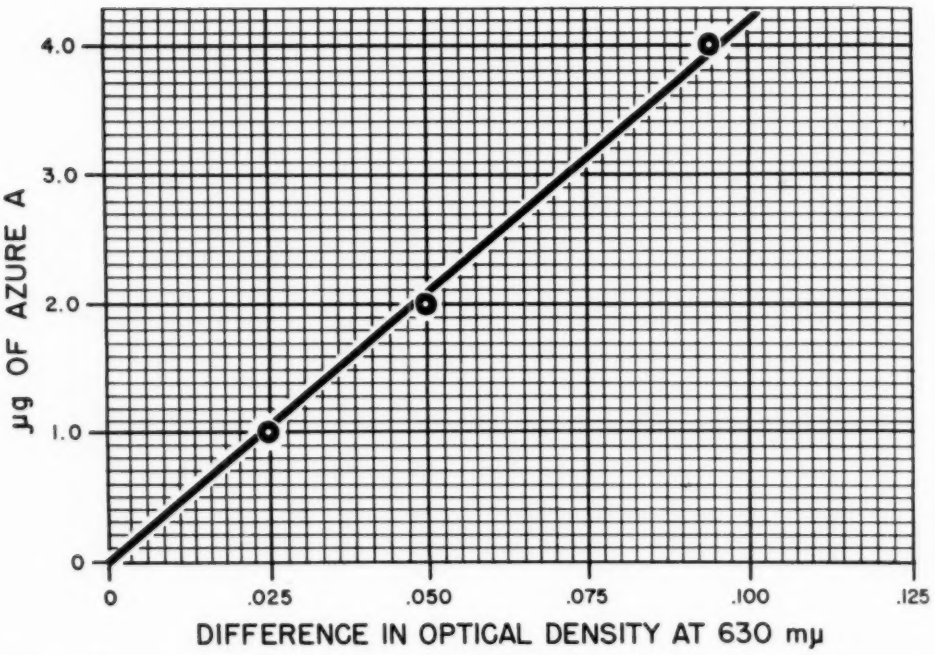

Fig. 1.—Comparison of delta optical density and micrograms of azure A per milliliter per pooled sera.

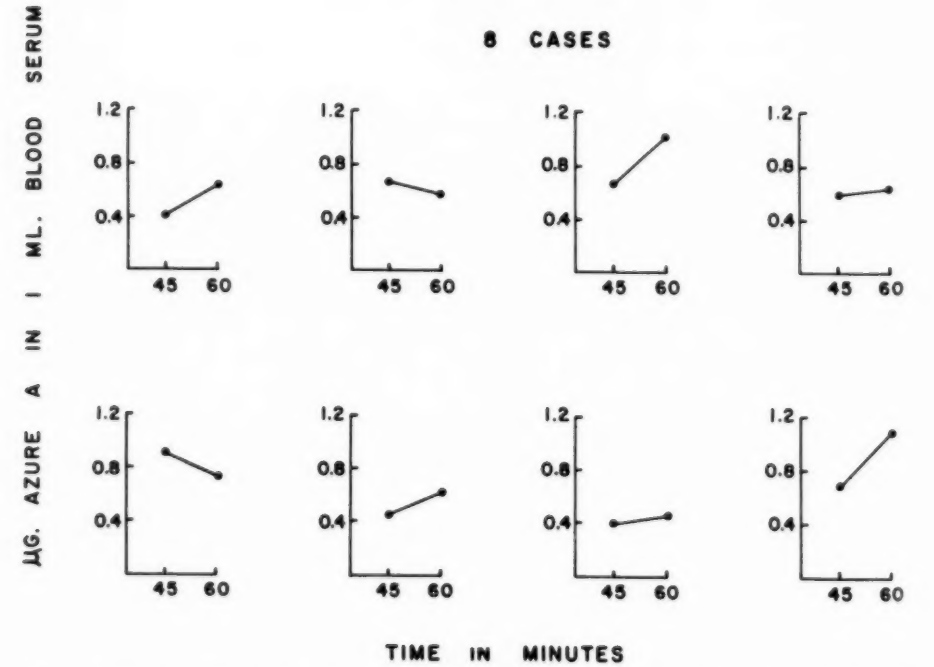

Fig. 2.—Micrograms of blood serum azure A, 45 and 60 minutes after oral administration of azure A resin compound.

Beckmann spectrophotometer* at 630 mμ with 0.035 mm. slit width. The optical densities of these aliquots are then redetermined after reduction of all the azure A to its leukoform by addition of at least 75 to 100 mg. (a pinch) of L-ascorbic acid and shaking.

The difference in optical density obtained before and after treatment with the ascorbic acid is used to calculate the level of azure A in the sample of the blood. The standard curve (Fig. 1) for this purpose was prepared by plotting the known amount of azure A in serial dilutions of a 4 mcg. per cent solution of azure A in pooled sera (Table I) treated with the copper sulfate–hydrochloric acid solution as already described against the differences in optical density of the azure A serum solutions before and after the addition of L-ascorbic acid.

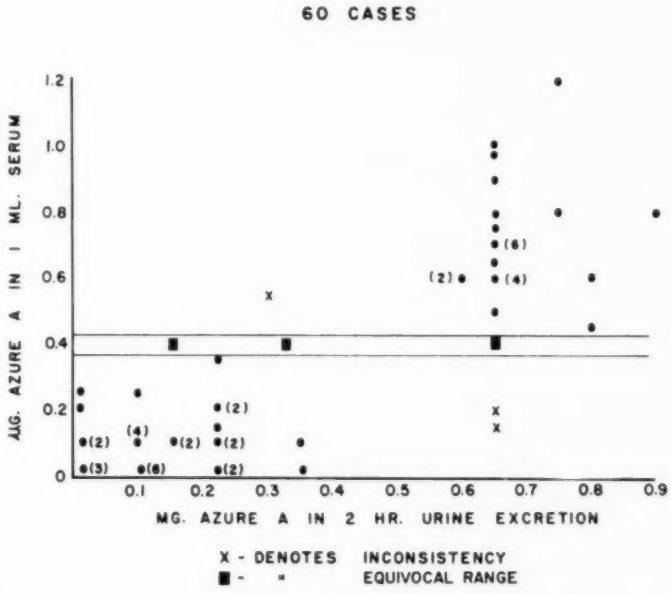

Fig. 3.—Comparison of azure A blood serum level with azure A urine excretion.

After the development of this technique, venous blood withdrawn at 45 and 60 minutes after the administration of the dye compound suggested that 50 minutes would be a possible optimal time for measuring the azure A level in the blood (Fig. 2).

The following procedure was used to compare the level of the azure A in the blood serum with the amount of azure A in the 2 hour urine:

The subject had no food from 11:00 P.M. on the night before the test was made until the test was completed. The first urine on arising was discarded and the individual then ingested 500 mg. caffeine sodium benzoate (2 tablets) with a glass of water. The azure A compound was taken 1 hour later. About 10 ml. of venous blood was drawn 50 minutes after the administration of the azure A compound; the total urine excreted during the 2 hour period after the ingestion of the dye compound was collected for measurement of the amount of azure A in the urine.

Measurement of Azure A in the Urine.—If the total urine collection is less than 300 ml. dilute with water to 300 ml. Pour 10 ml. aliquots of this urine into each of 2 test tubes and note color. To each aliquot add 1 drop of a solution containing 195 mg. $CuSO_4 \cdot 5H_2O$ in 100 ml. of 18 per cent HCl (6M). Place the test tubes in a boiling water bath for 10 minutes. Cool at room temperature for 2 hours. To urine aliquot add a pinch of L-ascorbic

*Any appropriate type of spectrophotometer can be used.

acid (about 300 mg.) and mix—this reduces the dye to a colorless form which allows this sample to be used as the control urine. Compare the color of both aliquots of urine with appropriate standards.[1]

TABLE I. PREPARATION OF SERIAL DILUTIONS FOR THE STANDARD CURVE*

DILUTION NUMBER (6 ML. VOL.)	SOLUTION OF AZURE A (ML. OF 4 MCG. %)	DISTILLED WATER (ML.)	AZURE A (MG./ML. OF DILUTION)
1	0.00	4.00	0.0
2	0.25	3.75	1.0
3	0.50	3.50	2.0
4	1.00	3.00	4.0

*Each test tube contains 1 ml. pooled sera, 1 ml. 0.5N HCl, and 1 drop of the $CuSO_4$-HCl solution.

Interpretation.—

Milligrams azure A in urine collection		Status of gastric acid secretion
0.6 or greater	signifies	free gastric HCl
less than 0.35	signifies	no free gastric HCl
0.35 to 0.6	signifies	borderline secretion

RESULTS

The scattergram (Fig. 3) provides a comparison between the azure A level of the blood serum withdrawn 50 minutes after the oral administration of the azure A resin compound and the milligrams of azure A in the total 2 hour urine excretion. In the 27 subjects secreting free acid as determined by the amount of azure A in the urine, the serum azure A level was greater than 0.4 μg in 24, exactly 0.4 μg in 1 individual, and between 0.1 and 0.4 μg in 2 subjects. Among the 33 subjects considered to be achlorhydric by the measurement of the azure A in the urine, the serum azure A level was 0 in 12, between 0.1 and 0.4 μg in 18, 0.4 μg in 2 individuals, and greater than 0.4 μg in 1 subject. Accepting any value over 0.4 μg of azure A in the 50 minute blood collection as an indication of the presence of free hydrochloric acid, there were 2 false negative tests (7.4 per cent) in the acid-secreting group and 1 false positive test among the achlorhydric individuals (3 per cent). At present a level of 0.4 μg is considered equivocal; there were 3 patients of the total group with such value.

DISCUSSION

These data suggest that the method described for measurement of the level of azure A in venous blood withdrawn 50 minutes after the administration of the azure A resin compound can determine the presence or absence of free hydrochloric acid with the azure A resin compound tubeless gastric analysis procedure. Further studies are now in progress both to check the optimum time of blood collection and to compare the level of azure A in the blood serum with the results of gastric acidity determined by direct intubation and by measurement of the amount of azure A in the 2 hour urine collection done on the same individual on the same day. Further investigations will determine whether the level of 0.4 μg will remain equivocal or signify the presence or absence of free hydrochloric acid. In the meantime, patients with this value should have gastric analysis by intubation if a repeat test remains equivocal.

Since the measurement of the blood serum azure A level is not as simple as that in the urine, the determination of azure A in the blood serum is recommended only for those individuals with urinary retention or in whom the urine collection is unreliable.

SUMMARY AND CONCLUSIONS

A method for the measurement of the azure A level in the blood serum is described. This allows the circumvention of the collection of urine with the tubeless gastric analysis procedure in individuals with urinary retention or in whom the collection of the urine is unreliable.

The technique consists of withdrawal of venous blood 50 minutes after the oral administration of the dye compound. Aliquots of the blood serum collection after treatment to change any colorless azure A to the oxidized color form are read on an appropriate spectrophotometer at 630 mμ. The difference between this optical density and that obtained after reduction of all the azure A to the leukoform by the addition of L-ascorbic acid is used to calculate the amount of azure A in the blood serum. The method of making appropriate standards for this purpose is described.

There were 2 false negative tests (7.4 per cent) and 1 false positive test (3 per cent) among the 60 individuals in whom the blood serum azure A level was compared with the state of gastric acidity, as measured by the amount of azure A in the 2 hour urine excretion. Three patients fell into the range designated as equivocal.

We gratefully acknowledge the advice and aid of Dr. Leon L. Miller and Mr. Martin Murray.

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THE CLINICAL USE OF TASCHDJIAN'S MEDIUM IN THE DIAGNOSIS OF VULVOVAGINAL CANDIDIASIS

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THE association of members of the Candida genus with the etiology of vulvovaginitis has been established since the work of Wilkinson in 1840.[35] In 1875, Houssman[22] proved the pathogenicity of *Candida albicans (Oidium albicans)* by infecting normal vaginal mucosa with material obtained from the discharge of other patients. Since these early studies, much has been written on the subject of vulvovaginal candidiasis, for it has proved to be one of the most common infections of the female genitals. In particular, vulvovaginal candidiasis is more prevalent where the contributing factors involve pregnancy, diabetes, or obesity.[10] In addition, chemical overtreatment during douching makes the vaginal mucosa much more susceptible to infection than it is normally.[3] There seems to be an increase in the incidence of all forms of candidiasis, particularly where patients have been treated with broad spectrum antibiotics, steroids, or by irradiation.[5] Such a situation points up the increased need for a relatively fast and reliable laboratory procedure as an aid in the diagnosis of candidiasis.

Accurate identification of *C. albicans* from among the yeasts commonly encountered in vaginal cultures is by no means as simple as is commonly assumed in routine diagnostic practice. Benham[5] emphasized that "No one criterion is sufficient for identification of the Candidas"; her statement remains valid at this time. Even serologic procedures have failed to distinctly separate all the species of Candida that are parasitic in man. Two of the most common species isolated from the human vagina, *C. albicans* and *Candida stellatoidea*,[19, 26, 51] can be differentiated serologically with the use of appropriately absorbed antisera, but as yet no one has reported a serologic procedure that will differentiate *C. albicans* from *Candida tropicalis*. Fermentation reactions are very valuable in characterizing the Candida species,[4, 5, 33, 55] but many degrees of variation have been reported,[56] so these criteria alone may fail to identify a species. Fermentation reactions are time consuming and cannot be used as rapid laboratory procedures in the diagnosis of candidiasis. The same criticism applies to animal pathogenicity tests. Gordon's[18] application of fluorescein-labeled specific antiserum in the differentiation of Candidas, both from culture and in clinical materials, is a procedure which embodies simplicity, rapidity, and specificity. However, the procedures involved in the production of the fluorescent antibodies are somewhat complicated,[11] so their routine use in clinical laboratories probably awaits their commercial availability.

Morphologic characteristics have been employed most widely for identification. Thus, the ability to produce chlamydospores has become a source of

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credence in identifying *C. albicans*, despite the fact that, occasionally, strains of *C. stellatoidea*,[5, 18, 39] *C. tropicalis*,[4, 5, 14, 39] and even *Candida krusei*,[14] which may play a role in infertility,[42] have shown this ability. Nevertheless, of the Candidas, only *C. albicans* has been seriously considered in the etiology of pruritus vulvae and vaginal discharge,[3, 19, 46] and the rapid diagnosis of *C. albicans* has, more often than not, depended upon the demonstration of chlamydospore formation. Many strains, otherwise identified as *C. albicans*, fail to produce chlamydospores on corn meal agar and other media formulated for this purpose.[17, 39, 45, 50] Table I indicates the variety of nutritionally deficient media that have been proposed to obviate this difficulty. The medium used in this study is Taschdjian's Cream of Rice infusion–Tween 80 agar. This medium stimulated the formation of chlamydospores in 17 strains of *C. albicans* isolated from oral thrush, skin lesions, or the feces of newborn infants, which had failed previously to form chlamydospores on a variety of chlamydospore media.[50] Taschdjian's medium was found, along with corn meal Tween 80 agar, to give the best results for rapid and abundant production of chlamydospores by *C. albicans* when compared with corn meal agar and Bacto chlamydospore agar.[27*]

The purpose of this study is to evaluate Taschdjian's medium clinically as a basis for a simple and relatively rapid one-step procedure leading to the

TABLE I. DIFFERENTIAL MEDIA PROPOSED FOR THE IDENTIFICATION OF CANDIDA ALBICANS

MEDIUM	DIFFERENTIATION OF CANDIDA ALBICANS BASED ON:	INVESTIGATORS	REFERENCE NO.
1. Corn meal agar	Chlamydospore production	Benham	4
2. Coagulated chicken plasma	Chlamydospore production	Fusillo and co-workers	15
3. Purified polysaccharide agar	Chlamydospore production	Nickerson and Mankowski	35
4. Polybismuth sulfite agar	Reduction of bismuth	Nickerson	34
5. Zein agar	Chlamydospore production	Reid and co-workers	40
6. Rice infusion agar	Chlamydospore production	Taschdjian	49
7. EMB agar + CO_2	Characteristic colony	Weld	53
8. Yellow corn meal agar	Chlamydospore production	Bakerspigel	1
9. Soil extract agar	Chlamydospore production	Bakerspigel	2
10. Buffered alkaline medium	Chlamydospore production	Liu and Newton	31
11. Dextrose peptone Tween 80* agar	Chlamydospore production	Seeliger	43
12. Littman's oxgall agar	Characteristic colony	Shapiro and co-workers	44
13. Potato and carrot pulp + CO_2	Chlamydospore production	Drouhet and Vieu	12
14. Potato and carrot pulp + bile	Chlamydospore production	Drouhet and Vieu	12
15. Blood agar + CO_2	Characteristic colony	Fahlberg and co-workers	14
16. Soluble starch agar	Chlamydospore production	Pollack and Benham	39
17. Rice Tween 80 agar	Chlamydospore production	Taschdjian	50
18. Corn meal Tween 80 agar	Chlamydospore production	Rosenthal	50
19. Nutrient medium + TTC† + neomycin	Reduction of TTC	Pagano and co-workers	36
20. Nutrient medium + phosphomolybdic acid	Reduction of molybdate	MacLaren and Armen	32

*Atlas Powder Co., Wilmington, Del.
†TTC = 2,3,5-triphenyltetrazolium chloride.

*Difco Laboratories, Detroit, Mich.

isolation and identification of *C. albicans* in confirmation of a clinical diagnosis of vulvovaginal candidiasis.

MATERIALS AND METHODS

Taschdjian's medium[59] was prepared as follows: 10 Gm. of Cream of Rice was sprinkled into 1,000 ml. of boiling tap water; this was boiled for 30 seconds, after which the suspension was filtered through cotton and the volume was reconstituted to 1,000 ml.; 10 Gm. of agar and 10 ml. of Tween 80 were added. The medium was autoclaved and was then poured into regular 90 mm. Petri dishes.

Specimen material for culturing was obtained in the following manner: a dry, self-retaining speculum was inserted into the vagina, after which a sterile, dry, cotton-tipped applicator was rotated over the vaginal wall and the vulva. The material so collected was inoculated directly onto the surface of 1 quadrant of a Taschdjian plate by rolling the swab over an area somewhat larger than that of the 22 mm. square cover glass used subse-

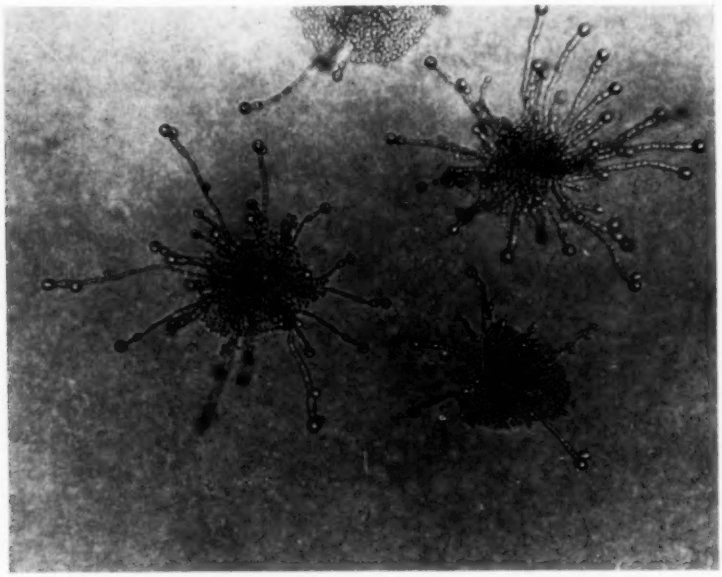

Fig. 1.—Chlamydospore production by young microcolonies of *C. albicans*. (Magnification ×150).

quently to cover most of the inoculation.[4, 28, 55] Cover glasses were rinsed in alcohol, drained, and flamed just prior to use. By such a procedure 4 specimens could be placed around the peripheral area of a plate. Incubation was at room temperature. An inoculated area was observed daily for chlamydospore formation by observation directly through the cover glass at the magnification of 100× using brightfield microscopy. Plates were observed for a period of 8 to 10 days where yeast-like fungi failed to produce chlamydospores.

Specimens were obtained from 200 gynecologic and obstetric outpatients of the University of Maryland Hospital Out-Patient Department who showed leukorrhea with or without subjective symptoms. Subjects with proved typical trichomonas vaginitis were excluded.

Patients with leukorrhea were categorized on clinical grounds as follows:

Group 1. Clinical candidiasis: all patients were placed in this group who exhibited the classical signs and symptoms of candidiasis, i.e., heavy, whitish, vaginal discharge, consisting often of cheese-like, curdy, and flaky material, frequently distributed in patches over the vaginal wall and to a lesser degree over the cervix; the vaginal mucosa grossly inflamed, reddish, and in varying degrees

edematous, often bleeding on light touch. These features lead to the most commonly encountered triad of complaints: pruritus vulvae et vaginae, dysuria, and dyspareunia.

Group 2. Presumptive candidiasis: with these patients the symptoms and findings were less conclusive or typical, but they presented a good possibility of having candidial infection.

Group 3. Previous unsuccessful therapy: this group was comprised of patients who for varying periods of time had been treated for vaginal discharge without significant relief or improvement.

Group 4. Miscellaneous: patients with apparent mixed infections; cervicitis, senile vaginitis, etc. were grouped in this category.

RESULTS

The microcolonies of yeast-like fungi could be observed through the cover glass within 24 hours in almost all cases where they constituted part of the vaginal flora. Pseudohyphal filaments might be seen to stem from the microcolonies at this time. The production of chlamydospores and blastospores on these pseudohyphae was subject to strain variation. In some cases *C. albicans* could be identified on Taschdjian's medium within 24 hours because of chlamydospore production. In other cases 5 to 6 days elapsed before these structures appeared; however, 3 to 4 days appeared to be the average time. Most strains appeared to produce chlamydospores predominantly with a diminution of blastospore production. Some strains produced voluminous numbers of blastospores and few chlamydospores. In other instances chlamydospores were produced fairly early in the incubation period, but were later completely masked by prodigious blastospore production. The latter possibility points up the value of daily observation of plates.

Figs. 1 and 2 illustrate chlamydospore production in young and older colonies, respectively, as seen directly through the cover glass overlying the inoculated area. Chlamydospore production very definitely was stimulated in the relatively anaerobic environment under the cover glass. Nevertheless, occasionally a strain was isolated which formed chlamydospores outside the cover glass area only.

Bacterial members of the vaginal flora were inhibited greatly on Taschdjian's medium; when overt growth by these microorganisms was present, however, it was easily distinguished microscopically. Although microscopic observation requires the removal of the cover of the Petri dish for varying periods of time, air contaminants were never a serious complication during the time alloted for culture study.

Our experience in determining the incidence of *C. albicans* in patients who exhibited leukorrhea is recorded in Table II. Of the 200 obstetric and gynecologic patients examined, 57 (28.5 per cent) harbored *C. albicans* in their vaginal flora. The incidence was greatest in Group 1 in which 30 (50.8 per cent) of the total of 59 patients who showed overt clinical candidiasis yielded the organism. Thirty (15 per cent) of 200 patients harbored yeast-like fungi that could not be identified as *C. albicans* by the method used. Well over one half of the patients were negative for yeast-like fungi altogether.

In Table III the data are categorized on the basis of pregnant and nonpregnant patients. Of the 67 who were pregnant, 36 (53.7 per cent) showed

TABLE II. INCIDENCE OF CANDIDA ALBICANS IN VULVOVAGINITIS USING TASCHDJIAN'S MEDIUM

GROUP ACCORDING TO CLINICAL DIAGNOSIS	NO. OF PATIENTS	MYCOLOGIC DIAGNOSIS					
		C. ALBICANS		YEAST-LIKE FUNGI NOT C. ALBICANS		NEGATIVE FOR YEAST-LIKE FUNGI	
		NO.	%	NO.	%	NO.	%
1. Candidiasis	59	30	50.8	7	11.8	22	37.3
2. Presumptive candidiasis	84	18	21.4	15	17.9	51	60.7
3. Unsuccessful therapy	20	4	20.0	4	20.0	12	60.0
4. Miscellaneous	37	5	13.5	4	10.8	28	75.7
Totals	200	57	28.5	30	15.0	113	56.5

the presence of *C. albicans;* 5 (7.5 per cent) harbored yeast-like fungi other than *C. albicans,* and 26 (38.8 per cent)gave negative cultures. Of 133 nonpregnant patients, *C. albicans* was isolated from 21 (15.8 per cent). Yeast-like fungi other than *C. albicans* were isolated from 25 (18.8 per cent), which incidence is 2.5 times greater than that recorded for the pregnant group. The data in Table III strongly suggest that *C. albicans* is more frequently associated with leukorrhea in pregnant than in nonpregnant women; in addition, it points up the apparent multiple etiology of clinical candidiasis.

TABLE III. INCIDENCE OF CANDIDA ALBICANS IN PREGNANT AND NONPREGNANT PATIENTS WITH LEUKORRHEA

STATUS	NO. OF PATIENTS	MYCOLOGIC DIAGNOSIS					
		C. ALBICANS		YEAST-LIKE FUNGI NOT C. ALBICANS		NEGATIVE FOR YEAST-LIKE FUNGI	
		NO.	%	NO.	%	NO.	%
Pregnant	67	36	53.7	5	7.5	26	38.8
Nonpregnant	133	21	15.8	25	18.8	87	65.4

DISCUSSION

Taschdjian's medium proved to be simple to use in clinical situations and yielded a high incidence of positive cultures. The diagnostic feature of chlamydospore formation appeared rapidly on the original plate inoculated directly with clinical material. The evaluation of a new diagnostic medium should logically also include a consideration of the efficiency with which it will demonstrate the etiologic agent in patients infected with, or harboring, the organism. Unfortunately, no absolute standard for comparison exists for vulvovaginitis caused by *C. albicans.* Marked discrepancies exist between clinical and laboratory diagnoses. Over the years, investigators, using a wide variety of techniques and diagnostic criteria, have diagnosed candidiasis in pregnant patients in the range of 15 to 43 per cent (Table IV). The incidence in gynecologic patients is significantly lower, and, although the data are sparse, the mean is very close to that recorded by Houssman in 1875,[22] 11 per cent. Despite the wide limits of the extremes in the references cited, remarkably similar data centering about the value of 30 per cent were recorded by about one half the investigators. In contrast, the incidence of *C. albicans* in pregnant patients with leukorrhea was found to be 53.7 per cent in this study, in which Taschdjian's medium was employed. While this higher incidence of positive cultures appears to speak well for the efficiency of Taschdjian's medium for the isolation of *C. albicans*

directly from this kind of clinical material, it would be premature to state that it is superior to other media. Certain obvious inherent sources of error exist in a comparison with data such as those in Table IV, where there were a variety of diagnostic criteria, the surveys varied quantitatively, and there was variation in incidence depending upon the socioeconomic level of the patients. In this regard Johnson and Mayne[25] did not uncover a racial predilection, but they did correlate increased incidence with a poor mode of living and poor personal hygiene.

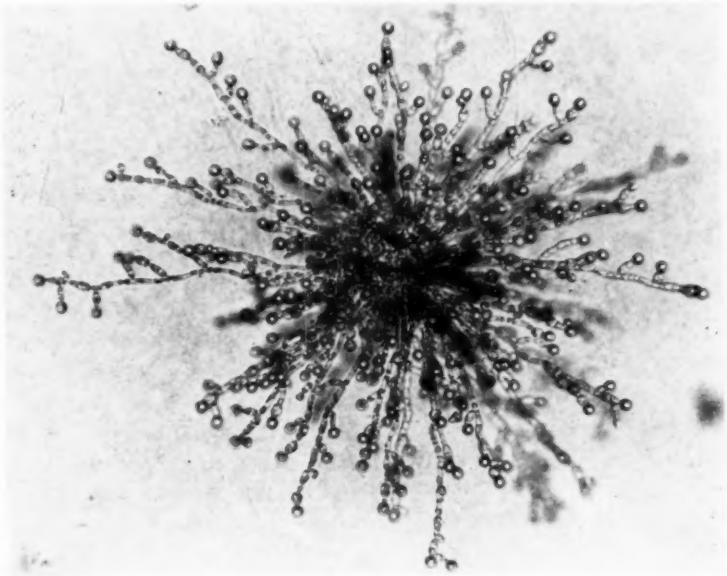

Fig. 2.—Increased chlamydospore production by an older microcolony of *C. albicans*. (Magnification ×150; reduced ¼.)

Although the high incidence of positive results suggests that Taschdjian's medium is efficient as a diagnostic tool for vulvovaginal candidiasis, comparative studies with other standard media will be required to establish beyond doubt that it possesses a significantly higher degree of efficiency in the isolation of *C. albicans*. Nevertheless, the simplicity of application and the rapidity with which morphologic structures useful for identification appear directly, without necessity for subculture, are sufficiently encouraging in themselves to warrant consideration of its routine use as a diagnostic medium.

One of the puzzling features of vulvovaginitis is the relative infrequency with which *C. albicans* is isolated. Even when the clinical picture is consistent with the classical description of candidiasis, this organism is isolated only in a fraction of the cases. This has been the experience of many investigators over the years (Table IV) employing a wide variety of media, and it was also true in this current study. Any one or all of several factors might contribute to this discrepancy between clinical and laboratory diagnosis of candidiasis: (1) the media employed may not permit the growth of all strains of *C. albicans* with high efficiency; (2) criteria for the identification of *C. albicans* may not be adequate; and/or (3) the clinical syndrome may have multiple etiologies.

TABLE IV. EXPERIENCE OF OTHER INVESTIGATORS REGARDING INCIDENCE OF VULVOVAGINAL CANDIDIASIS

INVESTIGATORS	REFERENCE NO.	YEAR	COUNTRY	PATIENTS	
				PREGNANT (%)*	NONPREGNANT (%)
Carter and Jones	7	1937	U.S.A.	36	14
Carter and co-workers	8	1940	U.S.A.	43	—
Castelaza-Ayala	9	1958	Mexico	30-40	—
Eastman	13	1950	U.S.A.	25	—
Halde and Aragon	20	1956	Philippines	39	—
Hesseltine and Beckette	21	1949	U.S.A.	25	—
Ikle	23	1958	Switzerland	30	—
Jackson	24	1956	U.S.A.	33	—
Johnson and Mayne	25	1948	U.S.A.	15	—
Lammert	29	1958	U.S.A.	30	10
Pandya et al.	37	1958	India	30	6
Rieben	41	1958	Switzerland	24	—
Seegar Jones	42	1955	U.S.A.	—	11
Stough and Blank	48	1958	U.S.A.	30	17
Weinstein and Wickerham	52	1938	U.S.A.	23	7
Woodruff and Hesseltine	57	1938	U.S.A.	28	—

*Percentages are to the nearest whole number.

The matter of plating efficieney of a medium inoculated directly from clinical material has already been presented. The criteria for identification of *C. albicans* bear reinvestigation. In the present study identification of *C. albicans* was based on chlamydospore formation. In the experience of Taschdjian,[49, 50] Kelly and Funigiello,[27] and in our own[47] this criterion has inspired a high degree of confidence. However, since strains of certain other Candida species have produced chlamydospores on rare occasions, their performance on Taschdjian's medium should be investigated thoroughly, using large numbers of strains. Eventually, the degree of correlation between chlamydospore production, fermentation characteristics, and antigenic structure should be established. In this regard, it is interesting to note that Winter and Foley[56] were unable to differentiate *C. albicans* and *C. stellatoidea* on the basis of chlamydospore production, fermentation tests, or pathogenicity tests. However, as noted earlier,[19, 26, 51] these 2 organisms can be separated on a serologic basis.

Regardless of the problems of differentiating *C. albicans* from other yeast-like fungi, the fact remains that in this study, in common with many others by different groups, no yeast-like fungus of any kind was isolated from a high proportion of patients with symptoms and signs compatible with candidiasis. Although deficiencies in the media and technique may contribute to this state of affairs, the consisteney with which this observation is made suggests that there may be other etiologic agents which operate in the majority of these cases. The relationship of various bacterial species to leukorrhea and vaginitis has generally received little definitive investigation. However, in recent years *Hemophilus vaginalis* has been studied in relation to these conditions, and some investigators feel it has a very definite etiologic role.[6, 16, 30]

SUMMARY AND CONCLUSIONS

1. Taschdjian's medium was evaluated clinically as the basis for a simple, one-step procedure which leads to the isolation and identification of *Candida albicans* in confirmation of a clinical diagnosis of vulvovaginal candidiasis. Chlamydospore production was the criterion for identifying *C. albicans*.

2. Cultures of yeast-like fungi were obtained from 87 (43.5 per cent) of 200 gynecologic and obstetric outpatients who shared the common symptom of leukorrhea; 57 (28.5 per cent) harbored *C. albicans* in their vaginal flora. Of 59 patients who were considered to have candidiasis clinically, 22 (37.3 per cent) had no yeast-like fungus infection.

3. Thirty-six (53.7 per cent) of 67 pregnant patients were infected with *C. albicans;* 21 (15.8 per cent) of 133 nonpregnant patients were also infected.

4. Taschdjian's medium, as a directly inoculated differential medium, could prove to have value in: confirming or negating a clinical diagnosis of vulvovaginal candidiasis; following the efficacy of therapeutic procedures; and elucidating the apparent multiple etiology of clinical vulvovaginal candidiasis.

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